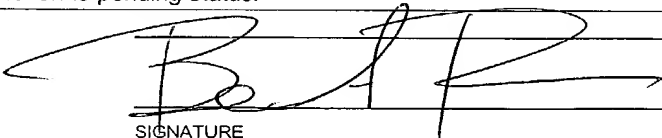


533 Rec'd PCT/PTO 22 AUG 2001

FORM PTO-1390 (Modified) (REV 5-93)		U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER	
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371				038602-1224	
				U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5) Unassigned 09/914042	
INTERNATIONAL APPLICATION NO. PCT/US00/04647		INTERNATIONAL FILING DATE 18 February 2000		PRIORITY DATE CLAIMED 22 February 1999	
TITLE OF INVENTION PYK2 BINDING PROTEINS					
APPLICANT(S) FOR DO/EO/US Sima LEV et al.					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1.	<input checked="" type="checkbox"/>	This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.			
2.	<input type="checkbox"/>	This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.			
3.	<input checked="" type="checkbox"/>	This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).			
4.	<input checked="" type="checkbox"/>	A proper Demand for International Preliminary Examination was made by the 19 <sup>th</sup> month from the earliest claimed priority date.			
5.	<input checked="" type="checkbox"/>	A copy of the International Application as filed (35 U.S.C. 371(c)(2))			
	<input type="checkbox"/>	is transmitted herewith (required only if not transmitted by the International Bureau).			
	<input checked="" type="checkbox"/>	has been transmitted by the International Bureau.			
	<input type="checkbox"/>	is not required, as the application was filed in the United States Receiving Office (RO/US)			
6.	<input type="checkbox"/>	A translation of the International Application into English (35 U.S.C. 371(c)(2)).			
7.	<input checked="" type="checkbox"/>	Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))			
	<input type="checkbox"/>	are transmitted herewith (required only if not transmitted by the International Bureau).			
	<input type="checkbox"/>	have been transmitted by the International Bureau.			
	<input type="checkbox"/>	have not been made; however, the time limit for making such amendments has NOT expired.			
	<input checked="" type="checkbox"/>	have not been made and will not be made.			
8.	<input type="checkbox"/>	A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).			
9.	<input type="checkbox"/>	An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).			
10.	<input checked="" type="checkbox"/>	A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).			
11.	<input type="checkbox"/>	Applicant claims small entity status under 37 CFR 1.27.			
Items 12. to 17. below concern other document(s) or information included:					
12.	<input type="checkbox"/>	An Information Disclosure Statement under 37 CFR 1.97 and 1.98.			
13.	<input type="checkbox"/>	An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.			
14.	<input type="checkbox"/>	A FIRST preliminary amendment.			
	<input type="checkbox"/>	A SECOND or SUBSEQUENT preliminary amendment.			
15.	<input type="checkbox"/>	A substitute specification.			
16.	<input type="checkbox"/>	A change of power of attorney and/or address letter.			
17.	<input type="checkbox"/>	Other items or information: OTHER			

U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.50) <b>Unassigned 09/014042</b>		INTERNATIONAL APPLICATION NO. PCT/US00/04647		ATTORNEY'S DOCKET NUMBER 038602-1224		
18. <input checked="" type="checkbox"/> The following fees are submitted:				<b>CALCULATIONS</b>		
Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EPO or JPO.....\$860.00						
International preliminary examination fee paid to USPTO (37 CFR 1.482).....\$690.00						
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) .....\$710.00						
Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$1,000.00						
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) .....\$100.00						
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$860.00		
Surcharge of \$130.00 for furnishing the oath or declaration later than 20 Months from the earliest claimed priority date (37 CFR 1.492(e))						
Claims	Number Filed		Included in Basic Fee		Extra Claims	Rate
Total Claims	36	-	20	=	16	x \$18.00
Independent Claims	11	-	3	=	8	x \$80.00
Multiple dependent claim(s) (if applicable)						\$270.00
TOTAL OF ABOVE CALCULATIONS =				\$1788.00		
Reduction by 1/2 for filing by small entity, if applicable.				\$0.00		
SUBTOTAL =				\$1788.00		
Processing fee of \$130.00 for furnishing English translation later the 20 months from the earliest claimed priority date (37 CFR 1.492(f)).				+		
TOTAL NATIONAL FEE =				\$1788.00		
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				+		
TOTAL FEES ENCLOSED =				\$1788.00		
				Amount to be:		
				refunded \$		
				charged \$		
<p>a. <input type="checkbox"/> A check in the amount of \$1788.00 to cover the above fees is enclosed.</p> <p>b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. <u>19-0741</u> in the amount of \$1788.00 to the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>19-0741</u>. A duplicate copy of this sheet is enclosed.</p>						
<p>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</p>						
<p>SEND ALL CORRESPONDENCE TO:</p> <p>Foley &amp; Lardner 3000 K Street, N.W., Ste. 500 Washington, DC 20007-5109 Telephone: 202-672-5475 Facsimile: 202-672-5399</p>						
				 SIGNATURE		
				NAME BETH A. BURROUS		
				REGISTRATION NUMBER 35,087		

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

Applicants: Joseph Schlessinger et al.  
Title: PYK2 BINDING PROTEINS  
Appl. No.: 09/914,042  
Appl. Filing Date: August 22, 2001  
Examiner: Unassigned  
Art Unit: Unassigned

**PRELIMINARY AMENDMENT**

Commissioner for Patents  
Box PATENT APPLICATION  
Washington, D.C. 20231

Sir:

Prior to examination of the present Application, Applicants respectfully request that the above-identified prior application be amended as follows:

**In the Specification:**

Please amend the specification as follows:

**On page 33**, delete the 2<sup>nd</sup> paragraph and replace the paragraph with the following in accordance with 37 CFR § 1.121. A marked up version showing changes is attached:

Figure 2 shows a multiple sequence alignment of Arf1 GAP, GCS1, and murine Pap regions that contain the zinc finger motif (SEQ ID NO. 8) CXXCX<sub>16</sub>CXXC, where X is any amino acid (residues 1-119, SEQ ID NO: 5; residues 5-122, SEQ ID NO: 6; and residues 243-381, SEQ ID NO: 7, respectively). Identical residues are framed and shadowed. The positions of four conserved cysteines of zinc finger motif (SEQ ID NO: 8) are marked by dots.

Atty. Dkt. No. 038602/1224

On page 44, delete the 2<sup>nd</sup> paragraph and replace the paragraph with the following in accordance with 37 CFR § 1.121. A marked up version showing changes is attached:

The amino terminus of Pap exhibits weak homology to  $\Delta$ -helical sequences of myosin and kinesin. It contains a predicted coiled-coil structure followed by a typical PH domain. The Arf GAP domain of Pap contains a typical CXXCX<sub>16</sub>CXXC zinc finger motif (SEQ ID NO. 8) identity to the zinc finger containing domains of Arf1 GAP and GCS1 proteins (Fig. 2). The proline rich sequence of Pap contains several consensus binding sites for type II SH3 domains (PXXP), including four binding sites for type II SH3 domains (PXXPXR) (Feng, et al., 1994. Science 266:1241-1247). Splicing out 45 amino acids from this region in Pap $\Delta$  results in PapE.

#### REMARKS

Applicants respectfully request that the foregoing amendments be made prior to examination of the present application.

Applicants believe that the present application is now in condition for allowance. Favorable consideration of the application as amended is respectfully requested.

The Examiner is invited to contact the undersigned by telephone if it is felt that a telephone interview would advance the prosecution of the present application.

Date March 18, 2002  
FOLEY & LARDNER  
Customer Number: 22428



22428

PATENT TRADEMARK OFFICE

Telephone: (202) 672-5475  
Facsimile: (202) 672-5399

Respectfully submitted,

By

Beth A. Burrous  
Attorney for Applicants  
Registration No. 35,087



**VERSION WITH MARKINGS TO SHOW CHANGES MADE****Marked up replacement paragraphs:****Page 33, 2<sup>nd</sup> paragraph:**

Figure 2 shows a multiple sequence alignment of Arf1 GAP, GCS1, and murine Pap regions that contain the zinc finger motif (SEQ ID NO: 8) CXXCX<sub>16</sub>CXXC, where X is any amino acid (residues 1-119, SEQ ID NO: 5; residues 5-122, SEQ ID NO: 6; and residues 243-381, SEQ ID NO: 7, respectively). Identical residues are framed and shadowed. The positions of four conserved cysteines of zinc finger motif (SEQ ID NO: 8) are marked by dots.

**Page 44, 2<sup>nd</sup> paragraph:**

The amino terminus of Pap exhibits weak homology to  $\Delta$ -helical sequences of myosin and kinesin. It contains a predicted coiled-coil structure followed by a typical PH domain. The Arf GAP domain of Pap contains a typical CXXCX<sub>16</sub>CXXC zinc finger motif (SEQ ID NO. 8) identity to the zinc finger containing domains of Arf1 GAP and GCS1 proteins (Fig. 2). The proline rich sequence of Pap contains several consensus binding sites for type II SH3 domains (PXXP), including four binding sites for type II SH3 domains (PXXPXR) (Feng, et al., 1994. Science 266:1241-1247). Splicing out 45 amino acids from this region in Pap $\Delta$  results in PapE.

09/914042  
Rec'd PCT/PTO 18 MAR 2002 #3

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

09/914042

Atty. Docket No: 038602/1224

In re patent application of

SCHLESSINGER, JOSEPH *et al.*

Serial No. 09/914,042

Filed: August 22, 2001

For: PYK2 BINDING PROTEINS

STATEMENT TO SUPPORT FILING AND SUBMISSION IN  
ACCORDANCE WITH 37 C.F.R. §§ 1.821-1.825

Assistant Commissioner for Patents  
Washington, D.C. 20231  
**Box SEQUENCE**

Sir:

In connection with a Sequence Listing submitted concurrently herewith, the undersigned hereby states that:

1. the submission, filed herewith in accordance with 37 C.F.R. § 1.821(g), does not include new matter;

2. the content of the attached paper copy and the attached computer readable copy of the Sequence Listing, submitted in accordance with 37 C.F.R. § 1.821(c) and (e), respectively, are the same; and

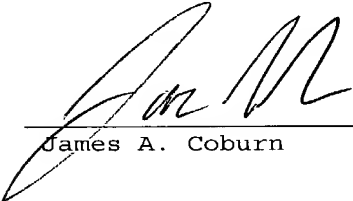
3. all statements made herein of their own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United

Serial No. 09/914,042

States Code and that such willful false statements may jeopardize the validity of the application or any patent resulting therefrom.

Respectfully submitted,

Dec. 10, 2001  
Date

  
James A. Coburn

**HARBOR CONSULTING**  
Intellectual Property Services  
1500A Lafayette Road  
Suite 262  
Portsmouth, N.H.  
800-318-3021

PYK2 BINDING PROTEINS

5

RELATED APPLICATIONS

The present application is related to U.S. applications  
Serial No. 08/357,642, by Lev and Schlessinger, Lyon & Lyon  
Docket No. 209/070, entitled "PYK2 related Products and  
Methods", filed December 15, 1994; Serial No. 08/460,626, by  
10 Lev and Schlessinger, Lyon & Lyon Docket No. 211/121, entitled  
"PYK2 related Products and Methods", filed June 2, 1995; and  
Serial No. 08/987,689 by Lev and Schlessinger, Lyon & Lyon  
Docket No. 230/110, entitled "PYK2 related Products and  
Methods", filed December 9, 1997, all of which are hereby  
15 incorporated by reference herein in their entirety including  
any drawings, figures, or tables.

FIELD OF THE INVENTION

The present invention relates to novel protein tyrosine  
20 kinase binding polypeptides, nucleotide sequences encoding  
such polypeptides, as well as various products and methods  
useful for the diagnosis and treatment of various kinase-  
related diseases and conditions.

25

BACKGROUND OF THE INVENTION

The following description of the background of the  
invention is provided to aid in understanding the invention,  
but is not admitted to be, or to describe, prior art to the  
invention.

30

Protein kinases are one of the largest families of  
eukaryotic proteins with several hundred known members. These

proteins share a 250-300 amino acid domain that can be subdivided into 12 distinct subdomains that comprise the common catalytic core structure. Multiple alignment of the sequences in the catalytic domain of protein kinases and subsequent parsimony analysis permits the segregation of related kinases into distinct branches of subfamilies including: tyrosine kinases, dual-specificity kinases, and serine/threonine kinases (STK's). Protein tyrosine kinases can be further divided into receptor and non-receptor classes by virtue of whether they possess or lack extracellular ligand-binding and transmembrane domains (Schlessinger, 1995. The Harvey Lectures Series 89:105-123).

On the basis of sequence similarity in the catalytic kinase domain and the presence of common structural motifs, numerous families of non-receptor tyrosine kinases have been defined (Neet, et al., 1996. Genes to Cells 1:147-169). Non-receptor tyrosine kinases may be recruited to the plasma membrane where they mediate cellular signaling by cell surface receptors lacking intrinsic protein tyrosine kinase activities. For instance, members of the Src family of protein tyrosine kinases are activated in response to stimulation of growth factor receptors and G-protein coupled receptors, as well as many other extracellular stimuli (Thomas, et al., 1997. Annu. Rev. Cell. Dev. Biol. 13:513-609). Focal adhesion kinase (FAK), on the other hand, plays a central role in integrin-mediated signaling (Sclapfer, et al., 1998. Trends Cell Biol. 8:151-157).

FAK and Pyk2 (proline-rich tyrosine kinase 2, also known as RAFTK, CAK $\beta$  and CADTK) comprise one family of PTKs (Sclapfer, et al., 1998. Trends Cell Biol. 8:151-157). Pyk2 and FAK exhibit approximately 45% amino acid identity and a

similar domain structure: a unique N-terminus, centrally located protein tyrosine kinase domain, and two proline-rich regions at the C-terminus. However, Pyk2 is not localized in focal contacts, but rather concentrated in the perinuclear region of cells.

Pyk2 is activated by a variety of extracellular signals that elevate intracellular calcium concentration (Lev, et al., 1995. Nature 376:737-45). In addition, treatments with phorbol esters or agonists of G-protein coupled receptors lead to Pyk2 tyrosine phosphorylation (Dikic, et al., 1996. Nature 383:547-550; Lev, et al., 1995. Nature 376:737-45; Li, et al., 1997. J. Biol. Chem. 272:14341-8). Pyk2, like FAK, can be regulated by activation of integrin receptors (Astier, et al., 1997. J. Biol. Chem. 272:19719-24; Li, et al., 1996. Blood 88:417-28).

The major autophosphorylation site of Pyk2 at tyrosine 402 functions as a docking site for the SH2 domain of Src. Pyk2, together with Src, functions as a link between heterotrimeric G-protein coupled receptors and the MAP kinase signaling pathway (Dikic, et al., 1996. Nature 383:547-550).

#### SUMMARY OF THE INVENTION

The instant invention relates, *inter alia*, to the cloning and characterization of a novel protein that specifically binds to Pyk2 designated Pap (Pyk2 C-terminus associated protein). In a preferred embodiment, the protein structure and partial or complete sequence is presented here, together with the classification, predicted or deduced protein structure, and an assessment of the pattern of expression of the Pap protein.

An association between Pap and Pyk2 is demonstrated, both *in vitro* and *in vivo*. Activation of Pyk2 leads to tyrosine phosphorylation of Pap. Endogenous Pap is also phosphorylated on tyrosine residues in response to phorbol ester stimulation.

Pap functions as an Arf GAP protein *in vivo*. Arf GAP proteins are involved in vesicular trafficking between the endoplasmic reticulum, golgi apparatus, and plasma membrane. Pap is localized in the plasma membrane, the cytoplasm and the Golgi compartment. When recruited to Golgi membranes, it can control Arf-mediated vesicle budding, since Pap functions as a substrate and downstream target for the protein tyrosine kinases Pyk2 and Src. Thus, these two protein kinases may be involved in regulation of some aspects of vesicular transport.

Thus, a first aspect of the invention features an isolated, enriched or purified nucleic acid molecule encoding a Pyk2 binding polypeptide. Preferably, the polypeptide is selected from the group consisting of Pap $\alpha$  and Pap $\beta$ .

By "isolated" in reference to nucleic acid is meant a polymer of 6 (preferably 21, more preferably 39, most preferably 75) or more nucleotides conjugated to each other, including DNA and RNA that is isolated from a natural source or that is synthesized. In certain embodiments of the invention, longer nucleic acids are preferred, for example those of 300, 600, 900 or more nucleotides and/or those having at least 50%, 60%, 75%, 90%, 95% or 99% identity to the sequence shown in SEQ ID NO:3; or SEQ ID NO:4.

The isolated nucleic acid of the present invention is unique in the sense that it is not found in a pure or separated state in nature. Use of the term "isolated" indicates that a naturally occurring sequence has been removed

from its normal cellular (i.e., chromosomal) environment. Thus, the sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only nucleotide chain present, but  
5 that it is essentially free (about 90-95% pure at least) of non-nucleotide material naturally associated with it, and thus is distinguished from isolated chromosomes.

By the use of the term "enriched" in reference to nucleic acid is meant that the specific DNA or RNA sequence  
10 constitutes a significantly higher fraction (2-5 fold) of the total DNA or RNA present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other DNA or RNA  
15 present, or by a preferential increase in the amount of the specific DNA or RNA sequence, or by a combination of the two. However, it should be noted that enriched does not imply that there are no other DNA or RNA sequences present, just that the relative amount of the sequence of interest has been  
20 significantly increased. The term "significant" is used to indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other nucleic acids of about at least 2-fold, more preferably at least 5- to 10-fold, or even more. The term  
25 also does not imply that there is no DNA or RNA from other sources. The other source DNA may, for example, comprise DNA from a yeast or bacterial genome, or a cloning vector such as pUC19. This term distinguishes from naturally occurring events, such as viral infection, or tumor type growths, in  
30 which the level of one mRNA may be naturally increased relative to other species of mRNA. That is, the term is meant



to cover only those situations in which a person has intervened to elevate the proportion of the desired nucleic acid.

It is also advantageous for some purposes that a nucleotide sequence be in purified form. The term "purified" in reference to nucleic acid does not require absolute purity (such as a homogeneous preparation). Instead, it represents an indication that the sequence is relatively more pure than in the natural environment (compared to the natural level this level should be at least 2-5 fold greater, e.g., in terms of mg/mL). Individual clones isolated from a cDNA library may be purified to electrophoretic homogeneity. The claimed DNA molecules obtained from these clones could be obtained directly from total DNA or from total RNA. The cDNA clones are not naturally occurring, but rather are preferably obtained via manipulation of a partially purified naturally occurring substance (messenger RNA). The construction of a cDNA library from mRNA involves the creation of a synthetic substance (cDNA) and pure individual cDNA clones can be isolated from the synthetic library by clonal selection of the cells carrying the cDNA library. Thus, the process, which includes the construction of a cDNA library from mRNA and isolation of distinct cDNA clones, yields an approximately  $10^6$ -fold purification of the native message. Thus, purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated.

By a "Pyk2 binding polypeptide" is meant 33 (preferably 40, more preferably 45, most preferably 55) or more contiguous amino acids set forth in the amino acid sequence of SEQ ID NO:1, or 2, or a functional derivative thereof as described

herein. In certain aspects, polypeptides of 100, 200, 300 or more amino acids are preferred. The Pyk2 binding polypeptide can be encoded by a full-length nucleic acid sequence, or any portion of the full-length nucleic acid sequence, so long as a functional activity of the polypeptide is retained. It is well known in the art that due to the degeneracy of the genetic code numerous different nucleic acid sequences can code for the same amino acid sequence. Equally, it is also well known in the art that conservative changes in amino acid can be made to arrive at a protein or polypeptide that retains the functionality of the original. In both cases, all permutations are intended to be covered by this disclosure. Other Pyk2 binding polypeptides can be identified using the methods described herein that were used to identify Pap $\alpha$  and Pap $\beta$ .

The amino acid sequence will be substantially similar to the sequence shown in SEQ ID NO:1 or SEQ ID NO:2, or fragments thereof. A sequence that is substantially similar to the sequence of SEQ ID NO:1 or SEQ ID NO:2 will preferably have at least 90% identity (more preferably at least 95% and most preferably 98-100%) to the sequence of SEQ ID NO:1 or SEQ ID NO:2 using a Smith-Waterman protein-protein search.

By "identity" is meant a property of sequences that measures their similarity or relationship. Generally speaking, identity is measured by dividing the number of identical residues by the total number of residues and gaps and multiplying the product by 100. "Gaps" are spaces in an alignment that are the result of additions or deletions of amino acids. Thus, two copies of exactly the same sequence have 100% identity, but sequences that are less highly conserved, and have deletions, additions, or replacements, may

have a lower degree of identity. Those skilled in the art will recognize that several computer programs are available for determining sequence identity.

In preferred embodiments, the invention features  
5 isolated, enriched, or purified nucleic acid molecules encoding a Pyk2 binding polypeptide comprising a nucleotide sequence that: (a) encodes a polypeptide having the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2; (b) is the complement of the nucleotide sequence of (a); (c) hybridizes  
10 under stringent conditions to the nucleotide molecule of (a) and encodes a naturally occurring Pyk2 binding polypeptide; (d) encodes a polypeptide having the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2, except that it lacks one or more, but not all, of the domains selected from the group  
15 consisting of N-terminal, coiled coil, PH, Arf GAP, Ankyrin, Proline-rich, SH3, and C-terminal domains; or (e) is the complement of the nucleotide sequence of (h).

The term "complement" refers to two nucleotides that can form multiple favorable interactions with one another. For  
20 example, adenine is complementary to thymine as they can form two hydrogen bonds. Similarly, guanine and cytosine are complementary since they can form three hydrogen bonds. A nucleotide sequence is the complement of another nucleotide sequence if all of the nucleotides of the first sequence are  
25 complementary to all of the nucleotides of the second sequence.

The term "domain" refers to a region of a polypeptide which contains a particular function. For instance, N-terminal or C-terminal domains of signal transduction proteins  
30 can serve functions including, but not limited to, binding molecules that localize the signal transduction molecule to

different regions of the cell or binding other signaling molecules directly responsible for propagating a particular cellular signal. Some domains can be expressed separately from the rest of the protein and function by themselves, while  
5 others must remain part of the intact protein to retain function. The latter are termed functional regions of proteins and also relate to domains.

The term "N-terminal domain" refers to the extracatalytic region located between the initiator methionine and the  
10 catalytic domain of the protein kinase. The N-terminal domain can be identified following a Smith-Waterman alignment of the protein sequence against the non-redundant protein database to define the N-terminal boundary of the catalytic domain.

Depending on its length, the N-terminal domain may or may not  
15 play a regulatory role in kinase function. An example of a protein kinase whose N-terminal domain has been shown to play a regulatory role is PAK65, which contains a CRIB motif used for Cdc42 and rac binding (Burbelo, P.D. et al. (1995) J. Biol. Chem. 270, 29071-29074).

20 The term "coiled coil domain" as used herein, refers to a polypeptide sequence that has a high probability of adopting a coiled-coil structure as predicted by computer algorithms such as COILS (Lupas, A. (1996) Meth. Enzymology 266:513-525).

Coiled-coils are formed by two or three amphipathic  $\Delta$ -helices  
25 in parallel. Coiled-coils can bind to coiled-coil domains of other polypeptides resulting in homo- or heterodimers (Lupas, A. (1991) Science 252:1162-1164). Coiled-coil-dependent oligomerization has been shown to be necessary for protein function including catalytic activity of serine/threonine  
30 kinases (Roe, J. et al. (1997) J. Biol. Chem. 272:5838-5845).

The term "proline-rich region" as used herein, refers to a region of a protein kinase whose proline content over a given amino acid length is higher than the average content of this amino acid found in proteins (*i.e.*, >10%). Proline-rich regions are easily discernable by visual inspection of amino acid sequences and quantitated by standard computer sequence analysis programs such as the DNASTar program EditSeq. Proline-rich regions have been demonstrated to participate in regulatory protein -protein interactions. Among these interactions, those that are most relevant to this invention involve the "PxxP" proline rich motif found in certain protein kinases (*i.e.*, human PAK1) and the SH3 domain of the adaptor molecule Nck (Galisteo, M.L. *et al.* (1996) *J. Biol. Chem.* 271:20997-21000). Other regulatory interactions involving "PxxP" proline-rich motifs include the WW domain (Sudol, M. (1996) *Prog. Biochys. Mol. Bio.* 65:113-132).

The term "C-terminal domain" generally refers to the region located after the last (located closest to the C-terminus) functional domain and including the carboxy-terminal amino acid residue of the Pky2 binding protein. By "functional" domain is meant any region of the polypeptide that may play a regulatory or catalytic role as predicted from amino acid sequence homology to other proteins or by the presence of amino acid sequences that may give rise to specific structural conformations (*e.g.* N-terminal domain). The C-terminal domain can be identified by using a Smith-Waterman alignment of the protein sequence against the non-redundant protein database to define the C-terminal boundary of the most C-terminal, functional domain. Depending on its length and amino acid composition, the C-terminal domain may

or may not play a regulatory role in Pky2 binding protein function.

The term "signal transduction pathway" refers to the molecules that propagate an extracellular signal through the cell membrane to become an intracellular signal. This signal can then stimulate a cellular response. The polypeptide molecules involved in signal transduction processes are typically receptor and non-receptor protein tyrosine kinases, receptor and non-receptor protein phosphatases, SRC homology 2 and 3 domains, phosphotyrosine binding proteins (SRC homology 2 (SH2) and phosphotyrosine binding (PTB and PH) domain containing proteins), proline-rich binding proteins (SH3 domain containing proteins), nucleotide exchange factors, and transcription factors.

Various low or high stringency hybridization conditions may be used depending upon the specificity and selectivity desired. These conditions are well-known to those skilled in the art. Under stringent hybridization conditions only highly complementary nucleic acid sequences hybridize. Preferably, such conditions prevent hybridization of nucleic acids having 1 or 2 mismatches out of 20 contiguous nucleotides.

By stringent hybridization assay conditions is meant hybridization assay conditions at least as stringent as the following: hybridization in 50% formamide, 5X SSC, 50 mM  $\text{NaH}_2\text{PO}_4$ , pH 6.8, 0.5% SDS, 0.1 mg/mL sonicated salmon sperm DNA, and 5X Denhart's solution at 42 °C overnight; washing with 2X SSC, 0.1% SDS at 45 °C; and washing with 0.2X SSC, 0.1% SDS at 45 °C.

In other preferred embodiments, the invention features isolated, enriched, or purified nucleic acid molecules encoding a Pyk2 binding polypeptide, further comprising a

vector or promoter effective to initiate transcription in a host cell. Preferably, the Pyk2 binding polypeptide is selected from the group consisting of PapA and PapE. The invention also features recombinant nucleic acid, preferably  
5 in a cell or an organism. The recombinant nucleic acid may contain a sequence set forth in SEQ ID NO:3 or SEQ ID NO:4, or a functional derivative thereof, and a vector or a promoter effective to initiate transcription in a host cell. The recombinant nucleic acid can alternatively contain a  
10 transcriptional initiation region functional in a cell, a sequence complementary to an RNA sequence encoding a Pyk2 binding polypeptide and a transcriptional termination region functional in a cell. Preferably the Pyk2 binding polypeptide is selected from the group consisting of PapA and PapE.

15 The term "vector" relates to a single or double-stranded circular nucleic acid molecule that can be transfected into cells and replicated within or independently of a cell genome. A circular double-stranded nucleic acid molecule can be cut and thereby linearized upon treatment with restriction  
20 enzymes. An assortment of nucleic acid vectors, restriction enzymes, and the knowledge of the nucleotide sequences cut by restriction enzymes are readily available to those skilled in the art. A nucleic acid molecule encoding a Pyk2 binding protein can be inserted into a vector by cutting the vector  
25 with restriction enzymes and ligating the two pieces together.

The term "transfecting" defines a number of methods to insert a nucleic acid vector or other nucleic acid molecules into a cellular organism. These methods involve a variety of techniques, such as treating the cells with high  
30 concentrations of salt, an electric field, detergent, or DMSO to render the outer membrane or wall of the cells permeable to

nucleic acid molecules of interest or use of various viral transduction strategies.

The term "promoter" as used herein, refers to a nucleic acid sequence needed for gene sequence expression. Promoter regions vary from organism to organism, but are well known to persons skilled in the art for different organisms. For example, in prokaryotes, the promoter region contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal synthesis initiation. Such regions will normally include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

In preferred embodiments, the isolated nucleic acid comprises, consists essentially of, or consists of the nucleic acid sequence set forth in SEQ ID NO:3 or SEQ ID NO:4, encodes the amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2, a functional derivative thereof, or at least 35, 40, 45, 50, 60, 75, 100, 200, or 300 contiguous amino acids of SEQ ID NO:1, or at least 10, 15, 20, 30, 40, 45, 50, 60, 75, 100, 200, or 300 contiguous amino acids of SEQ ID NO:2. The Pyk2 binding polypeptide comprises, consists essentially of, or consists of at least 35, 40, 45, 50, 60, 75, 100, 200, or 300 contiguous amino acids of SEQ ID NO:1, or at least 10, 15, 20, 30, 40, 45, 50, 60, 75, 100, 200, or 300 contiguous amino acids of SEQ ID NO:2. The nucleic acid may be isolated from a natural source by cDNA cloning or by subtractive hybridization. The natural source may be mammalian, preferably human, blood, semen, or tissue, and the nucleic acid may be synthesized by the triester method or by using an automated DNA synthesizer.



The term "mammal" refers preferably to such organisms as mice, rats, rabbits, guinea pigs, sheep, and goats, more preferably to cats, dogs, monkeys, and apes, and most preferably to humans.

5 In yet other preferred embodiments, the nucleic acid is a conserved or unique region, for example those useful for: the design of hybridization probes to facilitate identification and cloning of additional polypeptides, the design of PCR probes to facilitate cloning of additional polypeptides, 10 obtaining antibodies to polypeptide regions, and designing antisense oligonucleotides.

By "conserved nucleic acid regions", are meant regions present on two or more nucleic acids encoding a Pyk2 binding polypeptide, to which a particular nucleic acid sequence can 15 hybridize under lower stringency conditions. Examples of lower stringency conditions suitable for screening for nucleic acid encoding Pyk2 binding polypeptides are provided in Abe, et al. (J. Biol. Chem. 19:13361-13368, 1992), hereby incorporated by reference herein in its entirety, including 20 any drawings, figures, or tables. Preferably, conserved regions differ by no more than 5 out of 20 nucleotides.

By "unique nucleic acid region" is meant a sequence present in a nucleic acid coding for a Pyk2 binding polypeptide that is not present in a sequence coding for any 25 other naturally occurring polypeptide. Such regions preferably encode 33 (preferably 40, more preferably 45, most preferably 55) or more contiguous amino acids set forth in the amino acid sequence of SEQ ID NO:1, or 2. In particular, a unique nucleic acid region is preferably of mammalian origin.

30 A second aspect of the invention features a nucleic acid probe for the detection of nucleic acid encoding a Pyk2

binding polypeptide in a sample. Preferably, the polypeptide is selected from the group consisting of PapA and PapE. In preferred embodiments, the nucleic acid probe encodes a Pyk2 binding polypeptide that is a fragment of the protein encoded by the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2. The nucleic acid probe contains a nucleotide base sequence that will hybridize to the sequence set forth in SEQ ID NO:3 or SEQ ID NO:4, or a functional derivative thereof.

In other preferred embodiments, the nucleic acid probe hybridizes to nucleic acid encoding at least 35, 75, 90, 105, 120, 150, 200, 250, 300 or 350 contiguous amino acids of the full-length sequence set forth in SEQ ID NO:3, at least 12, 32, 75, 90, 105, 120, 150, 200, 250, 300 or 350 contiguous amino acids of the full-length sequence set forth in SEQ ID NO:4, or a functional derivative thereof.

Methods for using the probes include detecting the presence or amount of Pyk2 binding polypeptide RNA in a sample by contacting the sample with a nucleic acid probe under conditions such that hybridization occurs and detecting the presence or amount of the probe bound to the polypeptide's RNA. The nucleic acid duplex formed between the probe and a nucleic acid sequence coding for a Pyk2 binding polypeptide may be used in the identification of the sequence of the nucleic acid detected (Nelson et al., in *Nonisotopic DNA Probe Techniques*, Academic Press, San Diego, Kricka, ed., p. 275, 1992, hereby incorporated by reference herein in its entirety, including any drawings, figures, or tables). Kits for performing such methods may be constructed to include a container means having disposed therein a nucleic acid probe.

In a third aspect, the invention describes a recombinant cell or tissue comprising a nucleic acid molecule encoding a

Pyk2 binding polypeptide. Preferably, the polypeptide is selected from the group consisting of PapA and PapE. In such cells, the nucleic acid may be under the control of the genomic regulatory elements, or may be under the control of  
5 exogenous regulatory elements including an exogenous promoter. By "exogenous" it is meant a promoter that is not normally coupled *in vivo* transcriptionally to the coding sequence for the Pyk2 binding polypeptides.

The polypeptide is preferably a fragment of the protein  
10 encoded by the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2. By "fragment," is meant an amino acid sequence present in a Pyk2 binding polypeptide. Preferably, such a sequence comprises at least 35, 45, 50, 60, 100, 200, or 300 contiguous amino acids of the sequence set forth in SEQ ID  
15 NO:1, or at least 10, 15, 20, 30, 45, 50, 60, 100, 200, or 300 contiguous amino acids of the sequence set forth in SEQ ID NO:2.

In a fourth aspect, the invention features an isolated, enriched, or purified Pyk2 binding polypeptide. Preferably,  
20 the polypeptide is selected from the group consisting of PapA and PapE.

By "isolated" in reference to a polypeptide is meant a polymer of 6 (preferably 12, more preferably 18, most preferably 25, 32, 40, or 50) or more amino acids conjugated  
25 to each other, including polypeptides that are isolated from a natural source or that are synthesized. In certain aspects longer polypeptides are preferred, such as those with 100, 200, 300, 400, or more contiguous amino acids of the sequence set forth in SEQ ID NO:1, or SEQ ID NO:2.

30 The isolated polypeptides of the present invention are unique in the sense that they are not found in a pure or

separated state in nature. Use of the term "isolated" indicates that a naturally occurring sequence has been removed from its normal cellular environment. Thus, the sequence may be in a cell-free solution or placed in a different cellular environment. The term does not imply that the sequence is the only amino acid chain present, but that it is essentially free (about 90-95% pure at least) of non-amino acid-based material naturally associated with it.

By the use of the term "enriched" in reference to a polypeptide is meant that the specific amino acid sequence constitutes a significantly higher fraction (2-5 fold) of the total amino acid sequences present in the cells or solution of interest than in normal or diseased cells or in the cells from which the sequence was taken. This could be caused by a person by preferential reduction in the amount of other amino acid sequences present, or by a preferential increase in the amount of the specific amino acid sequence of interest, or by a combination of the two. However, it should be noted that enriched does not imply that there are no other amino acid sequences present, just that the relative amount of the sequence of interest has been significantly increased. The term significant here is used to indicate that the level of increase is useful to the person making such an increase, and generally means an increase relative to other amino acid sequences of about at least 2-fold, more preferably at least 5- to 10-fold or even more. The term also does not imply that there is no amino acid sequence from other sources. The other source of amino acid sequences may, for example, comprise amino acid sequence encoded by a yeast or bacterial genome, or a cloning vector such as pUC19. The term is meant to cover

only those situations in which man has intervened to increase the proportion of the desired amino acid sequence.

It is also advantageous for some purposes that an amino acid sequence be in purified form. The term "purified" in reference to a polypeptide does not require absolute purity (such as a homogeneous preparation); instead, it represents an indication that the sequence is relatively purer than in the natural environment. Compared to the natural level this level should be at least 2-5 fold greater (e.g., in terms of mg/mL). Purification of at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated. The substance is preferably free of contamination at a functionally significant level, for example 90%, 95%, or 99% pure.

In preferred embodiments, the Pyk2 binding polypeptide is a fragment of the protein encoded by the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2. Preferably, the polypeptide contains at least 35, 45, 50, 60, 100, 200, or 300 contiguous amino acids of the sequence set forth in SEQ ID NO:1, or at least 10, 15, 25, 35, 45, 50, 60, 100, 200, or 300 contiguous amino acids of the sequence set forth in SEQ ID NO:2, or a functional derivative thereof.

In preferred embodiments, the Pyk2 binding polypeptide comprises an amino acid sequence having: (a) the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2; (b) the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2, except that it lacks one or more, but not all, of the domains selected from the group consisting of N-terminal, coiled-coil, PH, Arf GAP, Ankyrin, Proline rich, SH3, and C-terminal domains.

The polypeptide can be isolated from a natural source by methods well-known in the art. The natural source may be mammalian, preferably human, blood, semen, or tissue, and the polypeptide may be synthesized using an automated polypeptide synthesizer.

In some embodiments the invention includes a recombinant Pyk2 binding polypeptide. Preferably the polypeptide is selected from the group consisting of PapA and PapE. By "recombinant Pyk2 binding polypeptide" is meant a polypeptide produced by recombinant DNA techniques such that it is distinct from a naturally occurring polypeptide either in its location (e.g., present in a different cell or tissue than found in nature), purity or structure. Generally, such a recombinant polypeptide will be present in a cell in an amount different from that normally observed in nature.

In a fifth aspect, the invention features an antibody (e.g., a monoclonal or polyclonal antibody) having specific binding affinity to a Pyk2 binding polypeptide. Preferably, the polypeptide is selected from the group consisting of PapA and PapE, or a domain or fragment of the polypeptide. Antibodies can be used to identify an endogenous source of Pyk2 binding polypeptides, to monitor cell cycle regulation, and for immuno-localization of Pyk2 binding polypeptides to the centrosomes.

By "specific binding affinity" is meant that the antibody binds to the target Pyk2 binding polypeptide with greater affinity than it binds to other polypeptides under specified conditions. Antibodies or antibody fragments are polypeptides that contain regions that can bind other polypeptides. The term "specific binding affinity" describes an antibody that

binds to a Pyk2 binding polypeptide with greater affinity than it binds to other polypeptides under specified conditions.

The term "polyclonal" refers to antibodies that are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen or an antigenic functional derivative thereof. For the production of polyclonal antibodies, various host animals may be immunized by injection with the antigen. Various adjuvants may be used to increase the immunological response, depending on the host species.

"Monoclonal antibodies" are substantially homogenous populations of antibodies to a particular antigen. They may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. Monoclonal antibodies may be obtained by methods known to those skilled in the art (Kohler et al., Nature 256:495-497, 1975, and U.S. Patent No. 4,376,110, both of which are hereby incorporated by reference herein in their entirety including any figures, tables, or drawings).

The term "antibody fragment" refers to a portion of an antibody, often the hypervariable region and portions of the surrounding heavy and light chains, that displays specific binding affinity for a particular molecule. A hypervariable region is a portion of an antibody that physically binds to the polypeptide target.

Antibodies or antibody fragments having specific binding affinity to a Pyk2 binding polypeptide of the invention may be used in methods for detecting the presence and/or amount of the polypeptide in a sample by probing the sample with the antibody under conditions suitable for Pyk2 binding polypeptide-antibody immunocomplex formation and detecting the

presence and/or amount of the antibody conjugated to the polypeptide. Diagnostic kits for performing such methods may be constructed to include antibodies or antibody fragments specific for the the polypeptide as well as a conjugate of a binding partner of the antibodies or the antibodies themselves.

An antibody or antibody fragment with specific binding affinity to a Pyk2 binding polypeptide of the invention can be isolated, enriched, or purified from a prokaryotic or eukaryotic organism. Routine methods known to those skilled in the art enable production of antibodies or antibody fragments, in both prokaryotic and eukaryotic organisms. Purification, enrichment, and isolation of antibodies, which are polypeptide molecules, are described above.

Antibodies having specific binding affinity to a Pyk2 binding polypeptide of the invention may be used in methods for detecting the presence and/or amount of the polypeptide in a sample by contacting the sample with the antibody under conditions such that an immunocomplex forms and detecting the presence and/or amount of the antibody conjugated to the polypeptide. Diagnostic kits for performing such methods may be constructed to include a first container containing the antibody and a second container having a conjugate of a binding partner of the antibody and a label, such as, for example, a radioisotope. The diagnostic kit may also include notification of an FDA approved use and instructions therefor.

In a sixth aspect, the invention features a hybridoma that produces an antibody having specific binding affinity to a Pyk2 binding polypeptide. Preferably, the polypeptide is selected from the group consisting of PapA and PapE. By "hybridoma" is meant an immortalized cell line that is capable



of secreting an antibody, for example an antibody to a polypeptide of the invention. In preferred embodiments, the antibody to the polypeptide comprises a sequence of amino acids that is able to specifically bind a Pyk2 binding polypeptide of the invention.

In a seventh aspect, the invention features an agent able to bind to a Pyk2 binding polypeptide. Preferably, the polypeptide is selected from the group consisting of PapA and PapE. The binding agent is preferably a purified antibody that recognizes an epitope present on a Pyk2 binding polypeptide of the invention. Other binding agents include molecules that bind to such polypeptides and analogous molecules that bind to Pyk2 binding polypeptides. Such binding agents may be identified by using assays that measure kinase binding partner activity, such as those that measure PDGFR activity. Binding agents to be tested include indolinones, quinazolines, quinoxalines, quinolines, and tyrphostins, examples of which and methods of making are described herein in Section VII.

The invention also features a method for screening for human cells containing a Pyk2 binding polypeptide of the invention or an equivalent sequence. The method involves identifying the novel polypeptide in human cells using techniques that are routine and standard in the art, such as those described herein for identifying the polypeptides of the invention (e.g., cloning, Southern or Northern blot analysis, *in situ* hybridization, PCR amplification, etc.). In addition the SOS recruitment system can be used *in vitro* to identify other Pyk2 binding polypeptides.

In an eighth aspect, the invention features methods for identifying a substance that modulates Pyk2 binding polypeptide activity comprising: (a) contacting a Pyk2 binding

polypeptide with a test substance; (b) measuring the activity of said polypeptide; and (c) determining whether said substance modulates the activity of said polypeptide.

Preferably, the Pyk2 binding polypeptide is selected from the  
5 group consisting of PapA and PapE.

Substances to be tested include indolinones, quinazolines, quinoxalines, quinolines, and tyrphostins, examples of which and methods of making are described herein in Section VII.

10 The term "modulates" refers to the ability of a substance to alter the function of a Pyk2 binding polypeptide of the invention. A modulator preferably activates, or more preferably inhibits, the activity of a polypeptide of the invention.

15 The term "activates" refers to increasing the cellular activity of the Pyk2 binding polypeptide. The term "inhibit" refers to decreasing the cellular activity of the polypeptide.

The term "modulates" also refers to altering the function of polypeptides of the invention by increasing or decreasing  
20 the probability that a complex forms between the polypeptide and a natural binding partner. A modulator preferably increases the probability that such a complex forms between the polypeptide and a natural binding partner, and most preferably decreases the probability that a complex forms  
25 between the polypeptide and a natural binding partner.

The term "complex" refers to an assembly of at least two molecules bound to one another. Signal transduction complexes often contain at least two protein molecules bound to one another. For instance, a protein tyrosine receptor protein  
30 kinase, GRB2, SOS, RAF, and RAS assemble to form a signal transduction complex in response to a mitogenic ligand.

The term "natural binding partner" refers to polypeptides, lipids, small molecules, or nucleic acids that bind to Pyk2 binding polypeptides in cells. A change in the interaction between a Pyk2 binding polypeptide and a natural binding partner can manifest itself as an increased or decreased probability that the interaction forms, or an increased or decreased concentration of polypeptide/natural binding partner complex. preferably the natural binding partner is selected from the group consisting of Pyk2 and Src, most preferably Pyk2.

The term "contacting" as used herein refers to mixing a solution comprising the test compound with a liquid medium bathing the cells of the methods. The solution comprising the compound may also comprise another component, such as dimethyl sulfoxide (DMSO), which facilitates the uptake of the test compound or compounds into the cells of the methods. The solution comprising the test compound may be added to the medium bathing the cells by utilizing a delivery apparatus, such as a pipet-based device or syringe-based device.

In a ninth aspect, the invention features methods for identifying a substance that modulates Pyk2 binding polypeptide activity in a cell comprising: (a) expressing Pyk2 binding polypeptide in a cell; (b) contacting said cell with a test substance; and (c) monitoring a change in cell phenotype or the interaction between said polypeptide and a natural binding partner. Preferably, the polypeptide is selected from the group consisting of PapA and PapE. Preferably the natural binding partner is selected from the group consisting of Pyk2 and Src. Most preferably the natural binding partner is Pyk2.

Substances to be tested include indolinones, quinazolines, quinoxalines, quinolines, and tyrphostins,

examples of which and methods of making are described herein in Section VII.

The term "expressing" as used herein refers to the production of Pyk2 binding polypeptides of the invention from  
5 a nucleic acid vector containing such polypeptide genes within a cell. The nucleic acid vector is transfected into cells using well known techniques in the art as described herein.

In a tenth aspect, the invention provides methods for treating a disease by administering to a patient in need of  
10 such treatment a substance that modulates the activity of a Pyk2 binding polypeptide. Preferably, the polypeptide is selected from the group consisting of PapA and PapE.

Substances useful for treatment of kinase-related disorders or diseases preferably show positive results in one  
15 or more *in vitro* assays for an activity corresponding to treatment of the disease or disorder in question. Examples of substances that can be screened for favorable activity are provided and referenced in section VII, below, and include indolinones, quinazolines, quinoxalines, quinolines, and  
20 tyrphostins. The substances that modulate the activity of the Pyk2 binding polypeptides preferably include, but are not limited to, antibodies, antisense oligonucleotides and inhibitors of Pyk2 binding polypeptides, as determined by methods and screens referenced in section VII and in the  
25 Examples sections, below. In preferred embodiments, the substance is an antisense oligonucleotide for the inhibition of the expression of Pyk2 binding polypeptide and fragments thereof. Preferably, the polypeptide is selected from the group consisting of PapA and PapE. Preferably, the antisense  
30 oligonucleotides of PapA and PapE are synthesized as phosphorothioates.

The term "preventing" refers to decreasing the probability that an organism contracts or develops an abnormal condition.

5 The term "treating" refers to having a therapeutic effect and at least partially alleviating or abrogating an abnormal condition in the organism.

The term "therapeutic effect" refers to the inhibition or activation factors causing or contributing to the abnormal condition. A therapeutic effect relieves to some extent one  
10 or more of the symptoms of the abnormal condition. In reference to the treatment of abnormal conditions, a therapeutic effect can refer to one or more of the following:  
(a) an increase in the proliferation, growth, and/or differentiation of cells; (b) inhibition (i.e., slowing or  
15 stopping) of cell death; (c) inhibition of degeneration; (d) relieving to some extent one or more of the symptoms associated with the abnormal condition; and (e) enhancing the function of the affected population of cells. Compounds demonstrating efficacy against abnormal conditions can be  
20 identified as described herein.

The term "abnormal condition" refers to a function in the cells or tissues of an organism that deviates from their normal functions in that organism. An abnormal condition can relate to cell proliferation, cell differentiation, or cell  
25 survival. Abnormal conditions include: cancer, cardiovascular, neurodegenerative, and immune disorders.

Abnormal cell proliferative conditions include cancers such as fibrotic and mesangial disorders, abnormal angiogenesis and vasculogenesis, wound healing, psoriasis,  
30 diabetes mellitus, and inflammation, as well as the cancers of the invention.

Abnormal differentiation conditions include, but are not limited to neurodegenerative disorders, slow wound healing rates, and slow tissue grafting healing rates.

Abnormal cell survival conditions relate to conditions in which programmed cell death (apoptosis) pathways are activated or abrogated. A number of kinase-related conditions are associated with the apoptosis pathways.

The term "aberration", in reference to the function of a Pyk2 binding polypeptide in a signal transduction process, refers to a Pyk2 binding polypeptide that is over- or under-expressed in an organism, mutated such that its activity is lower or higher than wild-type polypeptide activity, mutated such that it can no longer interact with a natural binding partner, is no longer modified by another binding molecule or protein phosphatase, or no longer interacts with a natural binding partner.

The term "administering" relates to a method of incorporating a compound into cells or tissues of an organism. The abnormal condition can be prevented or treated when the cells or tissues of the organism exist within the organism or outside of the organism. Cells existing outside the organism can be maintained or grown in cell culture dishes. For cells harbored within the organism, many techniques exist in the art to administer compounds, including (but not limited to) oral, parenteral, dermal, injection, and aerosol applications. For cells outside of the organism, multiple techniques exist in the art to administer the compounds, including (but not limited to) cell microinjection techniques, transformation techniques, and carrier techniques.

The abnormal condition can also be prevented or treated by administering a compound to a group of cells having an

aberration in a signal transduction pathway to an organism. The effect of administering a compound on organism function can then be monitored. The organism is preferably a mouse, rat, rabbit, guinea pig, or goat, more preferably a monkey or ape, and most preferably a human.

The eleventh aspect of the invention features methods for detection of nucleic acid encoding a Pyk2 binding polypeptide in a sample as a diagnostic tool for a disease or a disorder, wherein said method comprises: (a) contacting the sample with a nucleic acid probe which hybridizes under hybridization assay conditions to a nucleic acid target region of the Pyk2 binding polypeptide, the probe comprising the nucleic acid sequence encoding the binding polypeptide, fragments thereof, and the complements of the sequences and fragments; and (b) detecting the presence or amount of the probe:target region hybrid as an indication of the disease or the disorder. Preferably, the Pyk2 binding polypeptide is selected from the group consisting of PapA and PapE.

In preferred embodiments of the invention, the disease or disorder is selected from the group consisting of cancer, cardiovascular, neurodegenerative and immune disorders.

The nucleic acid "target region" is the full-length nucleotide base sequence set forth in SEQ ID NO:3, or SEQ ID NO:4, a functional derivative thereof, or a fragment thereof, to which the nucleic acid probe will specifically hybridize. Specific hybridization indicates that in the presence of other nucleic acids the probe only hybridizes detectably with the Pyk2 binding polypeptides of the invention's target region. Putative target regions can be identified by methods well known in the art consisting of alignment and comparison of the most closely related sequences in the database.

In preferred embodiments the nucleic acid probe hybridizes to a nucleic acid target region encoding at least 6, 12, 32, 50, 75, 90, 105, 120, 150, 200, 250, 300 or 350 contiguous amino acids of the sequence set forth in SEQ ID NO:1, or SEQ ID NO:2, or a functional derivative thereof. Hybridization conditions should be such that hybridization occurs only with the Pyk2 binding polypeptide genes, even in the presence of other nucleic acid molecules. Under stringent hybridization conditions only highly complementary nucleic acid sequences hybridize. Preferably, such conditions prevent hybridization of nucleic acids having 1 or 2 mismatches out of 20 contiguous nucleotides. Such conditions are defined *supra*.

The diseases for which detection of Pyk2 binding polypeptide genes in a sample could be diagnostic include diseases in which Pyk2 binding polypeptide nucleic acid (DNA and/or RNA) is amplified in comparison to normal cells. By "amplification" is meant increased numbers of polypeptide DNA or RNA in a cell compared with normal cells. In normal cells, Pyk2 binding polypeptides are typically found as single copy genes. In selected diseases, the chromosomal location of the polypeptide genes may be amplified, resulting in multiple copies of the gene, or amplification. Gene amplification can lead to amplification of such polypeptide RNA, or such polypeptide RNA can be amplified in the absence of the polypeptide's DNA amplification.

"Amplification" as it refers to RNA can be the detectable presence of polypeptide RNA in cells, since in some normal cells there is no basal expression of Pyk2 binding polypeptide RNA. In other normal cells, a basal level of expression of such polypeptide exists, therefore in these cases amplification is the detection of at least 1-2-fold, and



preferably more, such polypeptide RNA, compared to the basal level.

The diseases that could be diagnosed by detection of Pyk2 binding polypeptide nucleic acid in a sample preferably  
5 include cancer, cardiovascular, neurodegenerative and immune disorders. The test samples suitable for nucleic acid probing methods of the present invention include, for example, cells or nucleic acid extracts of cells, or biological fluids. The samples used in the above-described methods will vary based on  
10 the assay format, the detection method and the nature of the tissues, cells or extracts to be assayed. Methods for preparing nucleic acid extracts of cells are well known in the art and can be readily adapted in order to obtain a sample that is compatible with the method utilized.

15 The final aspect of the invention features antisense oligonucleotides for the inhibition of the expression of Pyk2 binding polypeptide. Preferably, the Pyk2 binding polypeptide is selected from the group consisting of PapA and PapE. In preferred embodiments, the oligonucleotide is the  
20 complement of a sequence encoding a fragment of the protein encoded by the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2. Preferably, the antisense oligonucleotides are synthesized as phosphorothionates.

Antisense oligonucleotides are preferably designed to  
25 specifically inhibit expression of a Pyk2 binding polypeptides. Preferably this is accomplished by identifying sequences unique to Pyk2 binding polypeptides of the invention (one or more). Preferably, these sequences are not present in other non-Pyk2 binding polypeptides present in a sample or in  
30 a patient. Methods to identify such sequences are well-known in the art and thus are only briefly described. Preferably,

antisense oligonucleotides are 10 to 100 oligonucleotides, more-preferably 15 to 30 nucleotides, and most preferably 18 to 25 nucleotides in length.

The antisense oligonucleotides of the invention are preferably used to inhibit Pyk2 binding protein expression in vivo in normal and tumor cells. Antisense oligonucleotides can be used either singly or in combination. Preferably, expression of a Pyk2 binding polypeptide is significantly reduced, and more preferably reduced to below the limit of detection. In preferred embodiments, treatment with the antisense oligonucleotides of the invention inhibits growth and/or induces apoptosis in cells. Antisense oligonucleotides can also be used to inhibit Pyk2 binding protein expression in human tumor cell xenografts in nude mice. In preferred  
15 embodiments, antisense oligonucleotides are used as a treatment in various human diseases and disorders in which Pyk2 binding polypeptides may be overexpressed or aberrantly expressed.

Additional antisense oligonucleotides and effective  
20 combinations can be identified by methods well known in the art. Briefly, cells or tissues overexpressing Pyk2 binding polypeptides can be contacted with antisense oligonucleotides, either singly or in combination, and the expression of the polypeptide's RNA, and/or the polypeptide itself can be  
25 determined by methods described herein. Preferably, treatment with antisense oligonucleotides to Pyk2 binding polypeptides will cause a decrease in the expression of such polypeptide RNA and/or polypeptide, more preferably expression is decreased significantly (1 to 2-fold), most preferably  
30 expression is decreased to an undetectable level.

The summary of the invention described above is not limiting and other features and advantages of the invention will be apparent from the following Detailed Description of the Invention, and from the Claims.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1a and 1b show the amino acid sequence and primary structure of the Pap isoforms. Figure 1a shows a schematic diagram of the primary structure of Pap isoforms.

5 Figure 1b shows the amino acid sequences of PapΔ (human; SEQ ID NO:1) and PapE (murine; SEQ ID NO:2) in single-letter code. The numbers represent positions of the amino acid residues. The predicted coiled-coil region (CoilScan program, GCG package) is enclosed in a rectangle with rounded corners. The PH domain is underlined with a double line. The Arf GAP domain is boxed. The Ankyrin homology region is underlined with a dashed line. The proline-rich region is underlined with solid line. The SH3 domain is also boxed.

Figure 2 shows a multiple sequence alignment of Arf1 GAP, GCS1, and murine Pap regions that contain the zinc finger motif CXXCX<sub>16</sub>CXXC, where X is any amino acid (residues 1-119, SEQ ID NO: 5; residues 5-122, SEQ ID NO:6; and residues 243-381, SEQ ID NO:7, respectively). Identical residues are framed and shadowed. The positions of four conserved cysteines of zinc finger motif are marked by dots.

Figure 3 shows a Northern blot analysis of Pap mRNA expression in various human tissues. Size markers in kilobase (Kb) are shown on the right of the figure. H, heart; B, brain; Pl, placenta; Lu, lungs; Li, liver; S, spleen; K, kidney; Pa, pancreas.

Figures 4a, 4b, 4c and 4d show the interaction between Pyk2 and Pap *in vitro*, in cultured cells, and in brain tissue. Figure 4a shows a Western blot of lysates from 293 cells transfected with expression vectors for Pyk2 or PKM, or vector alone. Lysates were subjected to SDS-PAGE immediately (total

lysate) or after immunoprecipitation (IP) with anti-Pyk2 antibodies, transferred to nitrocellulose filters, and blotted with a GST fusion protein containing the proline-rich region and the SH3 domain of Pap (3  $\mu$ g/mL) and anti-GST monoclonal antibodies (1:1000) (upper panel). The same filter was subsequently reprobed with anti-Pyk2 antibodies (lower panel). Arrows mark Pyk2/PKM. Positions of standard protein markers (kDa) are indicated on the right.

Figure 4b shows a Western blot of lysates from 293 cells transfected with expression vectors for Pyk2-HA and PapE, for PKM-HA and PapE, for PapE alone or for Pyk2-HA alone. Lysates were immunoprecipitated (IP) with anti-HA, anti-Pap or anti-Pyk2 antibodies. Immunoprecipitates were subjected to immunoblotting (IB) with anti-HA or anti-Pap antibodies. Arrows mark Pyk2/PKM and PapE. Positions of standard protein markers (kDa) are indicated on the right.

Figure 4c shows a Western blot of adult mouse brain homogenate. The homogenate was subjected to immunoprecipitation (IP) with anti-Pyk2 antibodies and immunoblotting (IB) with anti-Pap antibodies (right upper panel) or anti-Pyk2 antibodies (right lower panel). Lysates of PC12 cells infected with PapE virus were immunoprecipitated with anti-Pyk2 antibodies followed by immunoblotting (IB) with anti-Pap (left upper panel) or anti-Pyk2 (left lower panel) antibodies. Arrows mark Pap $\Delta$ , PapE, and PYK2. Positions of standard protein markers (kDa) are indicated on the right.

Fig. 4d shows a Western blot of lysates from 293 cells transfected with an expression vector for Src or for Pap $\Delta$  and Src. Lysates were immunoprecipitated (IP) with either preimmune (PI) or anti-Pap antibodies (PAP).

Immunoprecipitates were subjected to immunoblotting (IB) with anti-Src antibodies. Arrows mark Src and IgG heavy chain. Positions of standard protein markers (kDa) are indicated on the right.

5        Figures 5a, 5b and 5c depict tyrosine phosphorylation of Pap by Pyk2 and Src kinases. Figure 5a shows a Western blot of PC12 cells stimulated with the mixture of  $H_2O_2$  and  $Na_3VO_4$  (1 mM) for 20 min, or with PMA (2  $\mu$ M) for 10 min at 37 °C. Pap was immunoprecipitated from unstimulated (-) or stimulated (+) cells, immunoblotted (IB) with anti-pY antibodies (upper panels), and reprobed with anti-Pap antibodies (lower panels). Arrows mark Pap $\Delta$ . Positions of standard protein markers (kDa) are indicated on the right. Apart from endogenous Pap $\Delta$  (112 kDa), anti-Pap antibodies precipitated a band of apparent molecular weight of 140 kDa (lower panels) which may represent an additional uncharacterized PAP isoform expressed in PC12 cells.

10        Figure 5b shows a Western blot of lysates from 293 cells transfected with expression vectors for PapE and Pyk2-HA, PapE and PKM-HA, or for PapE alone. Lysates were subjected to immunoprecipitation with anti-Pap antibodies and immunoblotting with anti-pY antibodies. The same filters were reprobed with anti-Pap and anti-HA antibodies. Arrows mark Pyk2/PKM and PapE. Positions of standard protein markers (kDa) are indicated on the right.

25        Figure 5c shows a Western blot of lysates from 293 cells transfected with expression vectors for Pap $\Delta$  and Src, or for Pap $\Delta$  and Src(-), a kinase negative mutant of Src. Lysates were either separated by SDS-PAGE, immunoblotted with anti-pY antibodies and reprobed with anti-Pap or anti-Src antibodies,

30

or subjected to immunoprecipitation with anti-Pap antibodies and immunoblotting with anti-pY antibodies and anti-Pap antibodies. Arrows mark Pap $\Delta$  and Src. Positions of standard protein markers (kDa) are indicated on the right.

5        Figures 6a, 6b, 6c and 6d demonstrate Pap tyrosine phosphorylation by Pyk2 but not by FAK. Human 293 cells were transfected with 0.1, 1, or 10  $\mu$ g of expression vectors for PapE and Pyk2, or for PapE and FAK. Lysates from these cells were subjected to SDS-PAGE separation immediately (total  
10    lysate), or after immunoprecipitation (IP) with anti-Pap antibodies.

Figure 6 shows the result of immunoblotting with anti-PYK or anti-FAK antibodies. Figures 6b and 6d show the results of immunoblotting with anti-pY antibodies. Fig. 6c shows the  
15    results of immunoblotting with PAP antibodies. The tyrosine phosphorylated 112 kDa protein detected in total cell lysate (Fig. 6d) corresponds to Pyk2 and FAK as determined by reprobing the same filter with anti-Pyk2 or anti-FAK antibodies (not shown). Arrows mark Pyk2/FAK and PapE.  
20    Positions of standard protein markers (kDa) are indicated on the right.

Figures 7a and 7b demonstrate that recombinant Pap exhibits Arf GAP activity *in vitro*. Figure 7a is a graph of a single round of GTP hydrolysis on myristoylated Arf1 (open  
25    circles), myristoylated Arf5 (filled circles), myristoylated Arf6 (squares) and unmodified Arl2 (triangles). Hydrolysis was measured in the presence of crude phosphoinositides (1 mg/mL) as a source of PtdIns(4,5)P<sub>2</sub>, and the indicated concentrations of recombinant Pap. GAP activity is expressed  
30    as percentage of initially bound GTP hydrolyzed in 4 min. Figure 7b is a graph of a single round of GTP hydrolysis on

non-myristoylated Arf1. Hydrolysis was measured in the presence of 1.5 nM of recombinant Pap and the indicated phospholipids: none (no added phospholipid); PIP<sub>2</sub> (90  $\mu$ M phosphatidylinositol 4,5-bisphosphate); PA (750  $\mu$ M phosphatidic acid); PI (720  $\mu$ M phosphatidylinositol); PS (720  $\mu$ M phosphatidylserine); PC (720  $\mu$ M phosphatidylcholine). Error bars indicate SD values.

Fig. 8 shows an immunoblot of subcellular fractionated 293 cells overexpressing Pap $\Delta$ , fibroblast growth factor receptor (FGFR1, transmembrane protein), or Grb2 (cytosolic protein). Total (T), soluble (S), and particulate (P) fractions were separated on SDS-PAGE and immunoblotted with anti-Pap, anti-FGFR1, or anti-Grb2 antibodies.

Figure 9 shows immunofluorescent staining of HeLa cells transiently transfected with an expression vector for Pap $\Delta$ -myc. After 48h, the cells were fixed, permeabilized, labeled with anti-myc antibodies, stained with fluoresceine conjugated anti-myc antibodies and then examined with confocal microscope. Arrows mark Pap $\Delta$  localization in plasma membrane protrusions.

Figure 10 shows immunofluorescent staining of HeLa and COS-7 cells transiently transfected with Pap $\Delta$ -myc and Pyk2-HA expression vectors. After 48h, the cells were fixed, permeabilized, and double-labeled with anti-HA monoclonal antibodies and anti-Pap polyclonal antibodies. Following staining with fluoresceine conjugated anti-mouse IgG antibodies and rhodamine conjugated anti-rabbit IgG antibodies, the cells were examined by confocal microscopy. The images were superimposed (anaglyph) to detect areas of



overlapping localization. Arrows mark regions of Pap and Pyk2 colocalization at the plasma membrane.

Figure 11 shows COS-7 cells transiently transfected with expression vectors for Pap $\Delta$ -myc. After 48h, cells were fixed, permeabilized, and labeled with anti-myc, anti-mannosidase II or anti-E-COP antibodies. The cells were then stained with fluoresceine conjugated anti-mouse IgG antibodies and with rhodamine conjugated anti-rabbit IgG antibodies, and were examined by confocal microscopy. The images were superimposed (anaglyph) to detect areas of overlapping localization. Arrows indicate regions of Pap and mannosidase II or E-COP colocalization in the perinuclear area.

Figures 12a, 12b, 12c and 12d show inhibition of the generation of post-Golgi vesicles by Pap. Figure 12a is a graph of the percent release of VSV-G proteins. Assay mixtures containing Golgi fractions with [ $^{35}$ S] labeled sialylated VSV-G proteins, liver cytosol proteins and ATP were incubated at 37 °C for 1h with indicated concentrations of recombinant Pap. The reactions were stopped by chilling on ice. The radioactivity recovered in the supernatant after removal of residual Golgi membranes is expressed as a percentage of the initial radioactivity in the Golgi (release of VSV-G, %). Assay mixtures were incubated at 37 °C for 1h with (filled circles), or without (open circles) recombinant Pap (0.25 mg/mL). Figures 12b, 12c, and 12d are graphs of the percent of total vatroactivity in the preence of liver cytosol, with (Fig. 12b) or without (Fig. 12c) ATP (1 mM), or with GMP-PNP(100 IM) (Fig. 12d). Following incubation, the mixtures were chilled on ice and the released vesicles were separated in sucrose density gradients. The radioactivity

distribution in the gradient fractions, loading zone (S), and resuspended pellet (P), is expressed as a percentage of the total VSV-G radioactivity recovered in the gradient. Uncoated vesicles (fractions 2-5) sediment slower than coated ones (fractions 5-11).

Figure 13 shows a graph of the inhibition of SEAP secretion in 293 cells by overexpression of Pap. 293 cells were transfected with an expression vector for SEAP or co-transfected with expression vectors for SEAP and for Pap or Pyk2, or both. Brefeldin A (5  $\mu$ g/mL) was added to the medium for the duration of the assay. The amount of SEAP released into the medium is expressed as a percentage of total SEAP. All experiments were done three times in triplicate. Error bars represent SD values.

Figure 14 shows the nucleic acid sequence of Pap (SEQ ID NO:3).

Figure 15 shows the nucleic acid sequence of Pap (SEQ ID NO:4).

#### DETAILED DESCRIPTION OF THE INVENTION

This invention is drawn, *inter alia*, to the cloning and characterization of a novel protein that specifically binds to Pyk2, designated Pap (Pyk2 C-terminus associated protein).

Pap is comprised of an N-terminal  $\Delta$ -helical region with coiled-coil motif, a PH (pleckstrin homology) domain, a zinc finger containing Arf GAP domain, an ankyrin homology region, a proline-rich region and an SH3 domain. Pap and Pyk2 associate both *in vitro* and *in vivo*. Endogenous Pap is phosphorylated on tyrosine residues in response to phorbol-ester stimulation. Activation of Pyk2 also leads to tyrosine

phosphorylation of Pap. The closely related kinase FAK does not phosphorylate Pap.

Immunofluorescence analysis reveals that Pap is present in the plasma membrane, the cytoplasm, and the Golgi compartment. Addition of recombinant Pap strongly activates GTP hydrolysis on Arf *in vitro*, and inhibits Arf-dependent generation of post-Golgi vesicles. Moreover, overexpression of Pap in 293 cells downregulates constitutive secretion of a marker protein (SEAP).

Although the inventors do not wish to be limited to one theory, their current hypothesis is that Pap functions as an Arf GAP protein *in vivo*; recruitment of Pap to Golgi membranes controls Arf-mediated vesicle budding. Pap also functions as a substrate and downstream target for the protein tyrosine kinases Pyk2 and Src. Thus, the currently preferred hypothesis is that PYK2 and Src are involved in regulation of some aspects of vesicular transport through their interaction with Pap.

Pap was isolated by using the full length Pyk2 as bait in a yeast two hybrid screen. Northern blot analysis with a specific probe demonstrates that Pap mRNA is most abundant in the brain, in the kidney, and in the heart; lower expression is detected in the placenta, in the lungs and in the liver.

The amino terminus of Pap exhibits weak homology to  $\Delta$ -helical sequences of myosin and kinesin. It contains a predicted coiled-coil structure followed by a typical PH domain. PH domains function as membrane targeting signals and many PH domains bind specifically to phosphoinositides.

Immunofluorescence experiments demonstrate that a population of Pap molecules is localized at the plasma membrane (Figs. 8-11). Moreover, the PH domain of Pap complements the

temperature-sensitive *S. cerevisiae cdc25-2* (Aronheim, 1997. Nucleic Acids Research 25:3373-3374; Aronheim, et al., 1997. Mol. Cell. Biol. 17:3094-3102), permitting the yeast to grow at non-permissive temperatures, thereby demonstrating that the PH domain of Pap is targeted to the cell membrane. The inventors currently believe that the PH domain is probably responsible for targeting the protein to cell membranes.

The instant invention includes the cloning and characterization of two Pap isoforms, Pap $\Delta$  and PapE, that differ by the deletion of 45 amino acids from the proline rich region and 172 amino acids from the N-terminus of the protein. Using Pap-specific antibodies, several immunoreactive species were identified suggesting that additional isoforms of Pap may be generated by alternative splicing, all of which are intended to be included in the instant invention.

The GST fusion protein containing the SH3 domain of Pap binds to Pyk2 *in vitro*, suggesting that the interaction between Pyk2 and Pap is mediated by binding of the SH3 domain of Pap to the proline rich region in the C-terminus of Pyk2.

The proline-rich region of Pap $\Delta$  contains four putative binding sites for SH3 domains. One of the proline rich regions is spliced out to generate the PapE isoform. Another proline rich region is nearly identical to the canonical binding site for the SH3 domain of Src, suggesting that complex formation with Src is probably mediated by binding of the SH3 domain of Src to the proline-rich sequence of Pap.

Pyk2 forms a stable complex with Pap both *in vitro* and *in vivo*. Both of these proteins are localized in the plasma membrane (Figs. 8-11). In addition, endogenous Pap is tyrosine phosphorylated *in vivo* in response to stimulation with PMA, a well known activator of Pyk2 in different cell

types. In 293 cells Pap is tyrosine phosphorylated by Src or Pyk2. Tyrosine phosphorylation of Pap by Pyk2 or by the other protein tyrosine kinases may generate binding sites for SH2 domains of signaling proteins (Schlessinger, 1994. Curr. Opin. Gen. Dev. Biol. 4:25-30). Tyr470, for example, resides within a consensus binding site for the SH2 domain p85, the regulatory subunit of PI-3 kinase (PXXM).

The presence of an Arf GAP domain in Pap implies that Pap may contain an intrinsic GAP activity for Arf proteins. A recombinant protein composed of the PH domain, Arf GAP domain, and ankyrin homology region of Pap exhibited strong GAP activity towards Arf1 and Arf5 and weaker activity towards Arf6 (Fig. 7). The GAP activity was strictly dependent on the presence of PtdIns(4,5)P<sub>2</sub> in the assay mixture. A truncated protein lacking the PH domain did not have detectable GAP activity, suggesting that the PH domain may mediate membrane association of Pap, thereby allowing PtdIns(4,5)P<sub>2</sub>-dependent stimulation of Arf GAP activity.

Arf1 has been implicated in the control of vesicle transport in different intracellular compartments including the Golgi complex. The integrity of the Golgi complex and recruitment of coat proteins are dependent upon Arf1 activation. Overexpression of Arf1 GAP in cells causes disintegration of Golgi complex due to depletion of Arf-GTP associated with Golgi membranes (Aoe, et al., 1997. EMBOJ. 16:7305-16).

Immunofluorescence localization experiments demonstrate that Pap is localized in the Golgi compartment. Overexpression of Pap does not influence the integrity of the Golgi complex (Fig. 11C). However, post-Golgi vesicle release is inhibited in the presence of recombinant Pap protein and a

hydrolyzable nucleotide. Thus, inhibition is due to enhancement of the GTPase activity of an endogenous Arf protein associated with Golgi membranes (Fig. 12), suggesting that endogenous Arf1 proteins in Golgi membranes respond to  
5 Arf GAP activity of Pap.

Overexpression of Pap protein in 293 cells causes partial inhibition of constitutive secretion of SEAP, indicating that Pap may also exert similar Arf GAP activity in these cells. However, Pap-mediated reduction in SEAP secretion was not  
10 influenced by expression of Pyk2. This result is consistent with the cellular distribution of Pyk2 and Pap. Pap and Pyk2 are colocalized in the plasma membrane, but unlike Pap, Pyk2 is not found in the Golgi compartment. It appears therefore that the interaction between Pyk2 and Pap is restricted to the  
15 plasma membrane; Pap may interact with other tyrosine kinases in the Golgi complex.

#### I. The Nucleic Acids and Proteins of the Invention

A murine nucleic acid sequence identified by the SOS  
20 Recruitment System using PYK2 as bait, revealed an open reading frame of 783 amino acids with a predicted molecular weight of 88 kDa. The murine Pyk2 associated protein is designated PapE.

A human homolog, containing 5711 bases, was identified by  
25 searching the database with the murine sequence. The human homologue exhibits 95% sequence identity with the murine sequence, and contains 1006 amino acids with predicted molecular weight of 112 kDa. The human homologue, Pap $\Delta$ , is larger than the murine homologue, PapE, since it contains  
30 additional sequences that are probably generated by

alternative splicing. Pap $\Delta$  has 45 additional amino acids in the proline rich domain and 172 amino acids in the N-terminal domain (Fig. 1).

Analysis of the primary structures of murine and human  
5 sequences shows that Pap is a multidomain protein composed of several previously described sequence motifs. The N-terminus of Pap contains a unique amino acid sequence that is followed by a pleckstrin homology (PH) domain, an Arf GAP domain, three ankyrin repeats, a proline-rich region, and a SH3 domain (Fig.  
10 1).

The amino terminus of Pap exhibits weak homology to  $\Delta$ -helical sequences of myosin and kinesin. It contains a predicted coiled-coil structure followed by a typical PH domain. The Arf GAP domain of Pap contains a typical  
15 CXXCX<sub>16</sub>CXXC zinc finger sequence with high sequence identity to the zinc finger containing domains of Arf1 GAP and GCS1 proteins (Fig. 2). The proline rich sequence of Pap contains several consensus binding sites for SH3 domains (PXXP), including four binding sites for type II SH3 domains (PXXPXR)  
20 (Feng, et al., 1994. Science 266:1241-1247). Splicing out 45 amino acids from this region in Pap $\Delta$  results in PapE.

Pap is closely related to ASAP1, a protein that was recently cloned using Src as a bait in a yeast two hybrid screen (Brown, et al. Src. Mol. Cell. Biol., in press). Both  
25 Pap and ASAP1 contain a unique amino terminal sequence followed by a PH domain, Arf GAP domain, three ankyrin repeats, a proline rich sequence and an SH3 domain. However, Pap and ASAP1 exhibit different tissue expression patterns, and contain distinct proline-rich sequences suggesting  
30 interaction with divergent SH3 or WW domains containing signaling molecules. Pap and ASAP1 exhibit overall 68%

identity; the PH and Arf GAP domains are 69% identical, while the SH3 domains are 75% identical.

## II. Vesicular Trafficking

- 5        Src family kinases have been found associated with coated membrane regions in platelets (Stenberg, et al., 1997. Blood 89:2384-93). Src copurifies with synaptic vesicles in PC12 cells (Linstedt, et al., 1992. J. Cell Biol. 117:1077-84). Src associates with and phosphorylates several proteins
- 10    involved in membrane trafficking, such as the neuronal synaptic vesicle associated protein synapsin I, synaptophysin and synaptogyrin (Barnekow, et al., 1990. Oncogene 5:1019-24; Foster-Barber, et al., 1998. Proc. Natl. Acad. Sci. USA 95:4673-7; Janz, et al., 1998. J. Biol. Chem. 273:2851-7).
- 15        Vesicular traffic is controlled by different regulatory proteins including Ras-like GTPases (Rothman, et al., 1992. Nature 355:409-415), heterotrimeric G-proteins (Bomsel, et al., 1992. Mol. Biol. Cell. 3:1317-1328; Donaldson, et al., 1994. Curr. Opin. Cell. Biol. 6:527-32), phosphatidyl
- 20    inositol (PI)-transfer proteins (Bomsel, et al., 1992. Mol. Biol. Cell. 3:1317-1328; Donaldson, et al., 1994. Curr. Opin. Cell. Biol. 6:527-32; Ohashi, et al., 1995. Nature 377), PI-3 kinases (De Camilli, et al., 1996. Science 271:1533-9), phospholipase D (PLD; Exton, 1997. D.J. Biol. Chem.
- 25    272:15579-82), Ca<sup>2+</sup> influx (Goda, et al., 1997. Curr. Opin. Cell. Biol. 9:513-8), as well as by protein kinase C (PKC) (Buccione, et al., 1996. J. Biol. Chem. 271:3523-33; Simon, et al., 1996. J. Cell. Biol. 135:355-70). Heterotrimeric G-proteins may control vesicular transport through the Golgi
- 30    apparatus by regulating the activity of the small GTP binding protein ARF (ADP-ribosylation factor) that controls vesicle



formation (Bomsel, et al., 1992. Mol. Biol. Cell. 3:1317-1328).

Arfs were first isolated as cytosolic cofactors required for cholera toxin dependent ADP ribosylation of the G<sub>s</sub>A in in vitro assays (Kahn, et al., 1986. J. Biol. Chem. 261:7906-7911). This property, and the ability to rescue the lethal double *arf1-arf2* deletion in the yeast *Saccharomyces cerevisiae* distinguish Arfs from structurally similar Arf-like proteins (Arls; Kahn, et al., 1991. J. Biol. Chem. 266:2606-2614).

Six Arf family members have been so far identified. Arf1, the best characterized of the mammalian Arfs, functions in the recruitment of coat proteins to membranes of the Golgi apparatus either directly or through activation of PLD (Donaldson, et al., 1994. Curr. Opin. Cell. Biol. 6:527-32; Sabatini, et al., 1996. Biocell 20:287-300). It was shown that disruption of the Golgi complex by the fungal metabolite brefeldin A is due to inhibition of GDP to GTP exchange on Arf1 (Helms, et al., 1992. Nature 360:352-4). Arf1 has been also implicated in ER to Golgi transport, endosome function, and synaptic vesicle formation (Donaldson, et al., 1994. Curr. Opin. Cell. Biol. 6:527-32; Faundez, et al., 1998. Cell 93:423-32). Recent experiments provide evidence that Arf1 may physically associate with G-protein coupled receptors at the plasma membrane and enhance activation of PLD (Mitchell, et al., 1998. Nature 392:411-4). Arf6 is localized to the plasma membrane (Cavenagh, et al., 1996. J. Biol. Chem. 271:21767-74), where it appears to modulate both assembly of actin cytoskeleton and endocytosis (D'Souza-Schorey, et al., 1995. Science 267:1175-8; Radhakrishna, et al., 1997. J. Cell. Biol. 139:49-61).

Arfs are distinct from other small GTPases in their GTP-dependent binding to membranes and the absence of intrinsic GTPase activity. The completion of the GTPase cycle, therefore, requires interaction with guanine-nucleotide exchange factors (GEFs) and the GTPase-activating proteins (GAPs). Three Arf GEFs have been characterized, (Chardin, et al., 1996. Nature 384:481-4; Klarlund, et al., 1998. J. Biol. Chem. 273:1859-62; Meacci, et al., 1997. Proc. Natl. Acad. Sci. USA 94:1745-8).

10 Several Arf GAP activities from yeast (Poon, et al., 1996. Proc. Natl. Acad. Sci. USA 93:10074-7), bovine brain (Randazzo, et al., 1994. J. Biol. Chem. 269:10758-63) and rat liver (Makler, et al., 1995. J. Biol. Chem. 270:5232-7; Randazzo, 1997. Biochem J. 324:413-9) have been detected.

15 Acidic lipids and PtdIns(4,P)P<sub>2</sub> were found to be necessary cofactors for stimulation of GTP hydrolysis by certain Arf GAPs (Kahn, et al., 1996. J. Lipid Mediat. Cell. Signal. 14:209-14; Randazzo, 1997. J. Biol. Chem. 272:7688-92).

A recently identified Arf1-GAP protein contains a zinc finger sequence of approximately 120 amino acids terms Arf GAP domain (Cukierman, et al., 1995. Science 270:1999-2002). This part of Arf1 GAP exhibits a high degree of similarity to the *S. cerevisiae* family of "zinc finger" proteins. One of them, GCS1, mediates the transition from stationary phase to cell proliferation (Ireland, et al., 1994. EMBO J. 13:3812-3821), and has been shown to function as yeast Arf GAP in vitro and in vivo (Poon, et al., 1996. Proc. Natl. Acad. Sci. USA 93:10074-7).

30 III. Clinical applications of Novel Pyk2 binding polypeptides.

Pap polypeptides function as targets for the protein tyrosine kinases Pyk2 and Src and thus play a role in the signal transduction pathway. Additionally, Pap polypeptides function as Arf GAP proteins *in vivo* such that they control  
5 Arf-mediated vesicle budding when recruited to Golgi membranes. Consequently, the deregulation of Pap polypeptides through overexpression and/or mutational events may play a role in cancer, cardiovascular, neurodegenerative, and/or immune disorders through modulation of vesicular transport or  
10 signal transduction.

*In vitro* uses of Pap polypeptides include their use as diagnostic reagents if mutations in these genes are found to be associated with higher predisposition to cancer or other conditions. Such utility has proven valuable for genes such  
15 as p53, BRCA1, and in retinoblastoma (Fearson, E.R. (1997) Science, 278, 1043-1049 and Ponder, B. *Ibid*, 1050-1053). In addition, they may be used as a research reagent for the elucidation of biochemical pathways involved in signal transduction pathways and vesicular trafficking.

20

#### IV. Nucleic Acid Probes, Methods, and Kits for Detection of Pyk2 Binding Polypeptides

A nucleic acid probe of the present invention may be used  
25 to probe an appropriate chromosomal or cDNA library by usual hybridization methods to obtain other nucleic acid molecules of the present invention. A chromosomal DNA or cDNA library may be prepared from appropriate cells according to recognized methods in the art (cf. "Molecular Cloning: A Laboratory  
30 Manual", second edition, Cold Spring Harbor Laboratory, Sambrook, Fritsch, & Maniatis, eds., 1989).

In the alternative, chemical synthesis can be carried out in order to obtain nucleic acid probes having nucleotide sequences which correspond to N-terminal and C-terminal portions of the amino acid sequence of the polypeptide of interest. The synthesized nucleic acid probes may be used as primers in a polymerase chain reaction (PCR) carried out in accordance with recognized PCR techniques, essentially according to PCR Protocols, "A Guide to Methods and Applications", Academic Press, Michael, et al., eds., 1990, utilizing the appropriate chromosomal or cDNA library to obtain the fragment of the present invention.

One skilled in the art can readily design such probes based on the sequence disclosed herein using methods of computer alignment and sequence analysis known in the art ("Molecular Cloning: A Laboratory Manual", 1989, *supra*). The hybridization probes of the present invention can be labeled by standard labeling techniques such as with a radiolabel, enzyme label, fluorescent label, biotin-avidin label, chemiluminescence, and the like. After hybridization, the probes may be visualized using known methods.

The nucleic acid probes of the present invention include RNA, as well as DNA probes, such probes being generated using techniques known in the art. The nucleic acid probe may be immobilized on a solid support. Examples of such solid supports include, but are not limited to, plastics such as polycarbonate, complex carbohydrates such as agarose and sepharose, and acrylic resins, such as polyacrylamide and latex beads. Techniques for coupling nucleic acid probes to such solid supports are well known in the art.

The test samples suitable for nucleic acid probing methods of the present invention include, for example, cells

or nucleic acid extracts of cells, or biological fluids. The samples used in the above-described methods will vary based on the assay format, the detection method and the nature of the tissues, cells or extracts to be assayed. Methods for

5 preparing nucleic acid extracts of cells are well known in the art and can be readily adapted in order to obtain a sample that is compatible with the method utilized.

One method of detecting the presence of nucleic acids of the invention in a sample comprises (a) contacting said sample  
10 with the above-described nucleic acid probe under conditions such that hybridization occurs, and (b) detecting the presence of said probe bound to said nucleic acid molecule. One skilled in the art would select the nucleic acid probe according to techniques known in the art as described above.  
15 Samples to be tested include but should not be limited to RNA samples of human tissue.

A kit for detecting the presence of nucleic acids of the invention in a sample comprises at least one container means having disposed therein the above-described nucleic acid  
20 probe. The kit may further comprise other containers comprising one or more of the following: wash reagents and reagents capable of detecting the presence of bound nucleic acid probe. Examples of detection reagents include, but are not limited to radio-labeled probes, enzymatic labeled probes  
25 (horseradish peroxidase, alkaline phosphatase), and affinity labeled probes (biotin, avidin, or streptavidin). Preferably, the kit further comprises instructions for use.

A compartmentalized kit includes any kit in which reagents are contained in separate containers. Such  
30 containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allow the

efficient transfer of reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers include a container that accepts the test sample, a container that contains the probe or primers used in the assay, containers that contain wash reagents (such as phosphate buffered saline, Tris-buffers, and the like), and containers that contain the reagents used to detect the hybridized probe, bound antibody, amplified product, or the like. One skilled in the art will readily recognize that the nucleic acid probes described in the present invention can readily be incorporated into one of the established kit formats that are well known in the art.

V. DNA Constructs Comprising Pyk2 Binding Protein Nucleic Acid Molecules and Cells Containing These Constructs.

The present invention also relates to a recombinant DNA molecule comprising, 5' to 3', a promoter effective to initiate transcription in a host cell and the above-described nucleic acid molecules. In addition, the present invention relates to a recombinant DNA molecule comprising a vector and an above-described nucleic acid molecule. The present invention also relates to a nucleic acid molecule comprising a transcriptional region functional in a cell, a sequence complementary to an RNA sequence encoding an amino acid sequence corresponding to the above-described polypeptide, and a transcriptional termination region functional in said cell. The above-described molecules may be isolated and/or purified DNA molecules.

The present invention also relates to a cell or organism that contains an above-described nucleic acid molecule and thereby is capable of expressing a polypeptide. The polypeptide may be purified from cells that have been altered to express the polypeptide. A cell is said to be "altered to express a desired polypeptide" when the cell, through genetic manipulation, is made to produce a protein which it normally does not produce or which the cell normally produces at lower levels. One skilled in the art can readily adapt procedures for introducing and expressing either genomic, cDNA, or synthetic sequences into either eukaryotic or prokaryotic cells.

A nucleic acid molecule, such as DNA, is said to be "capable of expressing" a polypeptide if it contains nucleotide sequences which contain transcriptional and translational regulatory information and such sequences are "operably linked" to nucleotide sequences which encode the polypeptide. An operable linkage is a linkage in which the regulatory DNA sequences and the DNA sequence sought to be expressed are connected in such a way as to permit gene sequence expression. The precise nature of the regulatory regions needed for gene sequence expression may vary from organism to organism, but shall in general include a promoter region which, in prokaryotes, contains both the promoter (which directs the initiation of RNA transcription) as well as the DNA sequences which, when transcribed into RNA, will signal synthesis initiation. Such regions will normally include those 5'-non-coding sequences involved with initiation of transcription and translation, such as the TATA box, capping sequence, CAAT sequence, and the like.

If desired, the non-coding region 3' to the sequence encoding a kinase of the invention may be obtained by the above-described methods. This region may be retained for its transcriptional termination regulatory sequences, such as  
5 termination and polyadenylation. Thus, by retaining the 3'-region naturally contiguous to the DNA sequence encoding a Pyk2 binding polypeptide of the invention, the transcriptional termination signals may be provided. Where the transcriptional termination signals are not satisfactorily  
10 functional in the expression host cell, then a 3' region functional in the host cell may be substituted.

Two DNA sequences (such as a promoter region sequence and a sequence encoding a Pyk2 binding protein of the invention) are said to be operably linked if the nature of the linkage  
15 between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region sequence to direct the transcription of a gene sequence encoding a Pyk2 binding polypeptide of the invention, or (3) interfere with the  
20 ability of the gene sequence of a polypeptide of the invention to be transcribed by the promoter region sequence. Thus, a promoter region would be operably linked to a DNA sequence if the promoter were capable of effecting transcription of that DNA sequence. Thus, to express a gene encoding a Pyk2 binding  
25 polypeptide of the invention, transcriptional and translational signals recognized by an appropriate host are necessary.

The present invention encompasses the expression of a gene encoding a Pyk2 binding polypeptide of the invention (or  
30 a functional derivative thereof) in either prokaryotic or eukaryotic cells. Prokaryotic hosts are, generally, very



efficient and convenient for the production of recombinant proteins and are, therefore, one type of preferred expression system for polypeptides of the invention. Prokaryotes most frequently are represented by various strains of *E. coli*.

5 However, other microbial strains may also be used, including other bacterial strains.

In prokaryotic systems, plasmid vectors that contain replication sites and control sequences derived from a species compatible with the host may be used. Examples of suitable  
10 plasmid vectors may include pBR322, pUC118, pUC119 and the like; suitable phage or bacteriophage vectors may include  $\phi$ gt10,  $\phi$ gt11 and the like; and suitable virus vectors may include pMAM-neo, pKRC and the like. Preferably, the selected vector of the present invention has the capacity to replicate  
15 in the selected host cell.

Recognized prokaryotic hosts include bacteria such as *E. coli*, *Bacillus*, *Streptomyces*, *Pseudomonas*, *Salmonella*, *Serratia*, and the like. However, under such conditions, the polypeptide will not be glycosylated. The prokaryotic host  
20 must be compatible with the replicon and control sequences in the expression plasmid.

To express a Pyk2 binding polypeptide of the invention (or a functional derivative thereof) in a prokaryotic cell, it is necessary to operably link the sequence encoding the  
25 polypeptides of the invention to a functional prokaryotic promoter. Such promoters may be either constitutive or, more preferably, regulatable (i.e., inducible or derepressible). Examples of constitutive promoters include the *int* promoter of bacteriophage  $\phi$ , the *bla* promoter of the E-lactamase gene  
30 sequence of pBR322, and the *cat* promoter of the chloramphenicol acetyl transferase gene sequence of pPR325,

and the like. Examples of inducible prokaryotic promoters include the major right and left promoters of bacteriophage  $\phi$  ( $P_L$  and  $P_R$ ), the *trp*, *recA*, *OacZ*, *OacI*, and *gal* promoters of *E. coli*, the  $\Delta$ -amylase (Ulmanen et al., J. Bacteriol. 162:176-182, 1985) and the [-28-specific promoters of *B. subtilis* (Gilman et al., Gene Sequence 32:11-20, 1984), the promoters of the bacteriophages of *Bacillus* (Gryczan, In: The Molecular Biology of the Bacilli, Academic Press, Inc., NY, 1982), and *Streptomyces* promoters (Ward et al., Mol. Gen. Genet. 203:468-478, 1986). Prokaryotic promoters are reviewed by Glick (Ind. Microbiot. 1:277-282, 1987), Cenatiempo (Biochimie 68:505-516, 1986), and Gottesman (Ann. Rev. Genet. 18:415-442, 1984).

Proper expression in a prokaryotic cell also requires the presence of a ribosome-binding site upstream of the gene sequence-encoding sequence. Such ribosome-binding sites are disclosed, for example, by Gold et al. (Ann. Rev. Microbiol. 35:365-404, 1981). The selection of control sequences, expression vectors, transformation methods, and the like, are dependent on the type of host cell used to express the gene. As used herein, "cell", "cell line", and "cell culture" may be used interchangeably and all such designations include progeny. Thus, the words "transformants" or "transformed cells" include the primary subject cell and cultures derived therefrom, without regard to the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. However, as defined, mutant progeny have the same functionality as that of the originally transformed cell.

Host cells which may be used in the expression systems of the present invention are not strictly limited, provided that

they are suitable for use in the expression of the Pyk2 binding polypeptide of interest. Suitable hosts may often include eukaryotic cells. Preferred eukaryotic hosts include, for example, yeast, fungi, insect cells, mammalian cells  
5 either *in vivo*, or in tissue culture. Mammalian cells which may be useful as hosts include HeLa cells, cells of fibroblast origin such as VERO or CHO-K1, or cells of lymphoid origin and their derivatives. Preferred mammalian host cells include SP2/0 and J558L, as well as neuroblastoma cell lines such as  
10 IMR 332, which may provide better capacities for correct post-translational processing.

In addition, plant cells are also available as hosts, and control sequences compatible with plant cells are available, such as the cauliflower mosaic virus 35S and 19S, and nopaline  
15 synthase promoter and polyadenylation signal sequences. Another preferred host is an insect cell, for example the *Drosophila* larvae. Using insect cells as hosts, the *Drosophila* alcohol dehydrogenase promoter can be used (Rubin, Science 240:1453-1459, 1988). Alternatively, baculovirus  
20 vectors can be engineered to express large amounts of kinases of the invention in insect cells (Jasny, Science 238:1653, 1987; Miller et al., In: Genetic Engineering, Vol. 8, Plenum, Setlow et al., eds., pp. 277-297, 1986).

Any of a series of yeast expression systems can be  
25 utilized which incorporate promoter and termination elements from the actively expressed sequences coding for glycolytic enzymes that are produced in large quantities when yeast are grown in mediums rich in glucose. Known glycolytic gene sequences can also provide very efficient transcriptional  
30 control signals. Yeast provides substantial advantages in that it can also carry out post-translational modifications.

A number of recombinant DNA strategies exist utilizing strong promoter sequences and high copy number plasmids which can be utilized for production of the desired proteins in yeast.

Yeast recognizes leader sequences on cloned mammalian genes

5 and secretes peptides bearing leader sequences (*i.e.*, pre-peptides). Several possible vector systems are available for the expression of Pyk2 binding polypeptides of the invention in a mammalian host.

A wide variety of transcriptional and translational  
10 regulatory sequences may be employed, depending upon the nature of the host. The transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, cytomegalovirus, simian virus, or the like, where the regulatory signals are  
15 associated with a particular gene sequence which has a high level of expression. Alternatively, promoters from mammalian expression products, such as actin, collagen, myosin, and the like, may be employed. Transcriptional initiation regulatory signals may be selected which allow for repression or  
20 activation, so that expression of the gene sequences can be modulated. Of interest are regulatory signals which are temperature-sensitive so that by varying the temperature, expression can be repressed or initiated, or are subject to chemical (such as metabolite) regulation.

25 Expression of Pyk2 binding polypeptides of the invention in eukaryotic hosts requires the use of eukaryotic regulatory regions. Such regions will, in general, include a promoter region sufficient to direct the initiation of RNA synthesis. Preferred eukaryotic promoters include, for example, the  
30 promoter of the mouse metallothionein I gene sequence (Hamer *et al.*, J. Mol. Appl. Gen. 1:273-288, 1982); the TK promoter

of Herpes virus (McKnight, Cell 31:355-365, 1982); the SV40 early promoter (Benoist et al., Nature (London) 290:304-31, 1981); and the yeast gal4 gene sequence promoter (Johnston et al., Proc. Natl. Acad. Sci. (USA) 79:6971-6975, 1982; Silver et al., Proc. Natl. Acad. Sci. (USA) 81:5951-5955, 1984).

Translation of eukaryotic mRNA is initiated at the codon which encodes the first methionine. For this reason, it is preferable to ensure that the linkage between a eukaryotic promoter and a DNA sequence which encodes a polypeptide of the invention (or a functional derivative thereof) does not contain any intervening codons which are capable of encoding a methionine (i.e., AUG). The presence of such codons results either in the formation of a fusion protein (if the AUG codon is in the same reading frame as the polypeptide of the invention coding sequence) or a frame-shift mutation (if the AUG codon is not in the same reading frame as the polypeptide of the invention coding sequence).

A nucleic acid molecule encoding a Pyk2 binding polypeptide of the invention and an operably linked promoter may be introduced into a recipient prokaryotic or eukaryotic cell either as a nonreplicating DNA or RNA molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the gene may occur through the transient expression of the introduced sequence. Alternatively, permanent expression may occur through the integration of the introduced DNA sequence into the host chromosome.

A vector may be employed which is capable of integrating the desired gene sequences into the host cell chromosome. Cells which have stably integrated the introduced DNA into

their chromosomes can be selected by also introducing one or more markers which allow for selection of host cells which contain the expression vector. The marker may provide for prototrophy to an auxotrophic host, biocide resistance, e.g., antibiotics, or heavy metals, such as copper, or the like.

The selectable marker gene sequence can either be directly linked to the DNA gene sequences to be expressed, or introduced into the same cell by co-transfection. Additional elements may also be needed for optimal synthesis of mRNA.

These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. cDNA expression vectors incorporating such elements include those described by Okayama (Mol. Cell. Biol. 3:280-289, 1983).

The introduced nucleic acid molecule can be incorporated into a plasmid or viral vector capable of autonomous replication in the recipient host. Any of a wide variety of vectors may be employed for this purpose. Factors of importance in selecting a particular plasmid or viral vector include: the ease with which recipient cells that contain the vector may be recognized and selected from those recipient cells which do not contain the vector; the number of copies of the vector which are desired in a particular host; and whether it is desirable to be able to "shuttle" the vector between host cells of different species.

Preferred prokaryotic vectors include plasmids such as those capable of replication in *E. coli* (such as, for example, pBR322, ColE1, pSC101, pACYC 184,  $\Sigma$ VX; "Molecular Cloning: A Laboratory Manual", 1989, *supra*). *Bacillus* plasmids include pC194, pC221, pT127, and the like (Gryczan, In: The Molecular Biology of the Bacilli, Academic Press, NY, pp. 307-329, 1982). Suitable *Streptomyces* plasmids include p1J101 (Kendall

et al., J. Bacteriol. 169:4177-4183, 1987), and *Streptomyces* bacteriophages such as IC31 (Chater et al., In: Sixth International Symposium on Actinomycetales Biology, Akademiai Kiado, Budapest, Hungary, pp. 45-54, 1986). *Pseudomonas* plasmids are reviewed in John et al. (Rev. Infect. Dis. 8:693-704, 1986), and Izaki (Jpn. J. Bacteriol. 33:729-742, 1978).

Preferred eukaryotic plasmids include, for example, BPV, vaccinia, SV40, 2-micron circle, and the like, or their derivatives. Such plasmids are well known in the art

(Botstein et al., Miami Wntr. Symp. 19:265-274, 1982; Broach, In: "The Molecular Biology of the Yeast *Saccharomyces*: Life Cycle and Inheritance", Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470, 1981; Broach, Cell 28:203-204, 1982; Bollon et al., J. Clin. Hematol. Oncol. 10:39-48, 1980; Maniatis, In: Cell Biology: A Comprehensive Treatise, Vol. 3, Gene Sequence Expression, Academic Press, NY, pp. 563-608, 1980).

Once the vector or nucleic acid molecule containing the construct(s) has been prepared for expression, the DNA construct(s) may be introduced into an appropriate host cell by any of a variety of suitable means, i.e., transformation, transfection, conjugation, protoplast fusion, electroporation, particle gun technology, calcium phosphate-precipitation, direct microinjection, and the like. After the introduction of the vector, recipient cells are grown in a selective medium, which selects for the growth of vector-containing cells. Expression of the cloned gene(s) results in the production of a Pyk2 binding polypeptide of the invention, or fragments thereof. This can take place in the transformed cells as such, or following the induction of these cells to differentiate (for example, by administration of

bromodeoxyuracil to neuroblastoma cells or the like). A variety of incubation conditions can be used to form the peptide of the present invention. The most preferred conditions are those which mimic physiological conditions.

5

VI. Antibodies, Hybridomas, Methods of Use and Kits for Detection of Pyk2 Binding Polypeptides

The present invention relates to an antibody having  
10 binding affinity to a Pyk2 binding polypeptide of the invention. The polypeptide may have the amino acid sequence set forth in SEQ ID NO:1, SEQ ID NO:2, or a functional derivative thereof, or at least 9 contiguous amino acids thereof (preferably, at least 20, 30, 35, or 40 contiguous  
15 amino acids thereof).

The present invention also relates to an antibody having specific binding affinity to a Pyk2 binding polypeptide of the invention. Such an antibody may be isolated by comparing its binding affinity to a Pyk2 binding polypeptide of the  
20 invention with its binding affinity to other polypeptides. Those which bind selectively to a polypeptide of the invention would be chosen for use in methods requiring a distinction between a polypeptide of the invention and other polypeptides. Such methods could include, but should not be limited to, the  
25 analysis of altered Pyk2 binding polypeptide expression in tissue containing other polypeptides.

The Pyk2 binding polypeptides of the present invention can be used in a variety of procedures and methods, such as for the generation of antibodies, for use in identifying  
30 pharmaceutical compositions, and for studying DNA/protein interaction.



The Pyk2 binding polypeptides of the present invention can be used to produce antibodies or hybridomas. One skilled in the art will recognize that if an antibody is desired, such a peptide could be generated as described herein and used as an immunogen. The antibodies of the present invention include monoclonal and polyclonal antibodies, as well fragments of these antibodies, and humanized forms. Humanized forms of the antibodies of the present invention may be generated using one of the procedures known in the art such as chimerization or CDR grafting.

The present invention also relates to hybridomas that produce the above-described monoclonal antibodies, or binding fragment thereof. A hybridoma is an immortalized cell line that is capable of secreting a specific monoclonal antibody.

In general, techniques for preparing monoclonal antibodies and hybridomas are well known in the art (Campbell, "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology," Elsevier Science Publishers, Amsterdam, The Netherlands, 1984; St. Groth et al., J. Immunol. Methods 35:1-21, 1980). Any animal (mouse, rabbit, and the like) which is known to produce antibodies can be immunized with the selected polypeptide. Methods for immunization are well known in the art. Such methods include subcutaneous or intraperitoneal injection of the polypeptide. One skilled in the art will recognize that the amount of polypeptide used for immunization will vary based on the animal that is immunized, the antigenicity of the polypeptide and the site of injection.

The polypeptide may be modified or administered in an adjuvant in order to increase the peptide antigenicity. Methods of increasing the antigenicity of a polypeptide are

well known in the art. Such procedures include coupling the antigen with a heterologous protein (such as globulin or E-galactosidase) or through the inclusion of an adjuvant during immunization.

5 For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/0-Agl4 myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells. Any one of a number of methods well known in the art can be used to identify the  
10 hybridoma cell that produces an antibody with the desired characteristics. These include screening the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz et al., Exp. Cell Res. 175:109-124, 1988). Hybridomas secreting the desired antibodies are cloned and the class and  
15 subclass are determined using procedures known in the art (Campbell, "Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology", *supra*, 1984).

For polyclonal antibodies, antibody-containing antisera  
20 is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures. The above-described antibodies may be detectably labeled. Antibodies can be detectably labeled through the use of radioisotopes, affinity  
25 labels (such as biotin, avidin, and the like), enzymatic labels (such as horse radish peroxidase, alkaline phosphatase, and the like) fluorescent labels (such as FITC or rhodamine, and the like), paramagnetic atoms, and the like. Procedures for accomplishing such labeling are well-known in the art, for  
30 example, see Stemberger et al., J. Histochem. Cytochem. 18:315, 1970; Bayer et al., Meth. Enzym. 62:308-319, 1979;

Engval et al., Immunol. 109:129-135, 1972; Goding, J. Immunol. Meth. 13:215-226, 1976. The labeled antibodies of the present invention can be used for *in vitro*, *in vivo*, and *in situ* assays to identify cells or tissues that express a specific peptide.

The above-described antibodies may also be immobilized on a solid support. Examples of such solid supports include plastics such as polycarbonate, complex carbohydrates such as agarose and sepharose, acrylic resins such as polyacrylamide and latex beads. Techniques for coupling antibodies to such solid supports are well known in the art (Weir et al., "Handbook of Experimental Immunology" 4th Ed., Blackwell Scientific Publications, Oxford, England, Chapter 10, 1986; Jacoby et al., Meth. Enzym. 34, Academic Press, N.Y., 1974). The immobilized antibodies of the present invention can be used for *in vitro*, *in vivo*, and *in situ* assays as well as in immunochromatography.

Furthermore, one skilled in the art can readily adapt currently available procedures, as well as the techniques, methods and kits disclosed herein with regard to antibodies, to generate peptides capable of binding to a specific peptide sequence in order to generate rationally designed antipeptide peptides (Hurby et al., "Application of Synthetic Peptides: Antisense Peptides", In Synthetic Peptides, A User's Guide, W.H. Freeman, NY, pp. 289-307, 1992; Kaspczak et al., Biochemistry 28:9230-9238, 1989).

Anti-peptide peptides can be generated by replacing the basic amino acid residues found in the peptide sequences of the Pyk2 binding polypeptide of the invention with acidic residues, while maintaining hydrophobic and uncharged polar groups. For example, lysine, arginine, and/or histidine

residues are replaced with aspartic acid or glutamic acid and glutamic acid residues are replaced by lysine, arginine or histidine.

The present invention also encompasses a method of  
5 detecting a Pyk2 binding polypeptide in a sample, comprising:  
(a) contacting the sample with an above-described antibody,  
under conditions such that immunocomplexes form, and (b)  
detecting the presence of said antibody bound to the  
polypeptide. In detail, the methods comprise incubating a  
10 test sample with one or more of the antibodies of the present  
invention and assaying whether the antibody binds to the test  
sample. Altered levels of a polypeptide of the invention in a  
sample as compared to normal levels may indicate disease.

Conditions for incubating an antibody with a test sample  
15 vary. Incubation conditions depend on the format employed in  
the assay, the detection methods employed, and the type and  
nature of the antibody used in the assay. One skilled in the  
art will recognize that any one of the commonly available  
immunological assay formats (such as radioimmunoassays,  
20 enzyme-linked immunosorbent assays, diffusion based  
Ouchterlony, or rocket immunofluorescent assays) can readily  
be adapted to employ the antibodies of the present invention.  
Examples of such assays can be found in Chard ("An  
Introduction to Radioimmunoassay and Related Techniques"  
25 Elsevier Science Publishers, Amsterdam, The Netherlands,  
1986), Bullock et al. ("Techniques in Immunocytochemistry,"  
Academic Press, Orlando, FL Vol. 1, 1982; Vol. 2, 1983; Vol.  
3, 1985), Tijssen ("Practice and Theory of Enzyme  
Immunoassays: Laboratory Techniques in Biochemistry and  
30 Molecular Biology," Elsevier Science Publishers, Amsterdam,  
The Netherlands, 1985).

The immunological assay test samples of the present invention include cells, protein or membrane extracts of cells, or biological fluids such as blood, serum, plasma, or urine. The test samples used in the above-described method will vary based on the assay format, nature of the detection method and the tissues, cells or extracts used as the sample to be assayed. Methods for preparing protein extracts or membrane extracts of cells are well known in the art and can readily be adapted in order to obtain a sample which is testable with the system utilized.

A kit contains all the necessary reagents to carry out the previously described methods of detection. The kit may comprise: (i) a first container means containing an above-described antibody, and (ii) second container means containing a conjugate comprising a binding partner of the antibody and a label. Preferably, the kit also contains instructions for use. In another preferred embodiment, the kit further comprises one or more other containers comprising one or more of the following: wash reagents and reagents capable of detecting the presence of bound antibodies.

Examples of detection reagents include, but are not limited to, labeled secondary antibodies, or in the alternative, if the primary antibody is labeled, the chromophoric, enzymatic, or antibody binding reagents that are capable of reacting with the labeled antibody. The compartmentalized kit may be as described above for nucleic acid probe kits. One skilled in the art will readily recognize that the antibodies described in the present invention can readily be incorporated into one of the established kit formats that are well known in the art.

VII. Isolation of Compounds Which Interact With Pyk2 Binding  
Polypeptides

The present invention also relates to a method of  
5 detecting a compound capable of binding to a Pyk2 binding  
polypeptide of the invention, comprising incubating the  
compound with a polypeptide of the invention and detecting the  
presence of the compound bound to the polypeptide. The  
compound may be present within a complex mixture, for example,  
10 serum, body fluid, or cell extracts.

The present invention also relates to a method of  
detecting an agonist or antagonist of Pyk2 binding polypeptide  
activity or of such Pyk2 binding polypeptide binding partner  
activity, comprising incubating cells that produce a Pyk2  
15 binding polypeptide of the invention in the presence of a  
compound and detecting changes in the level of Pyk2 binding  
polypeptide activity or Pyk2 binding polypeptide binding  
partner activity. The compounds thus identified would produce  
a change in activity indicative of the presence of the  
20 compound. The compound may be present within a complex  
mixture, for example, serum, body fluid, or cell extracts.  
Once the compound is identified it can be isolated using  
techniques well known in the art.

The present invention also encompasses a method of  
25 agonizing (stimulating) or antagonizing kinase associated  
activity in a mammal comprising administering to said mammal  
an agonist or antagonist to a kinase of the invention in an  
amount sufficient to effect said agonism or antagonism. A  
method of treating diseases in a mammal with an agonist or  
30 antagonist of Pyk2 binding polypeptide activity comprising  
administering the agonist or antagonist to a mammal in an

amount sufficient to agonize or antagonize Pyk2 binding polypeptide associated functions is also encompassed in the present application.

In an effort to discover novel treatments for diseases, 5 biomedical researchers and chemists have designed, synthesized, and tested molecules that inhibit the function of protein kinases. Some small organic molecules form a class of compounds that modulate the function of protein kinases. Examples of molecules that have been reported to inhibit the 10 function of protein kinases include, but are not limited to, bis monocyclic, bicyclic or heterocyclic aryl compounds (PCT WO 92/20642, published November 26, 1992 by Maguire et al.), vinylene-azaindole derivatives (PCT WO 94/14808, published July 7, 1994 by Ballinari et al.), 1-cyclopropyl-4-pyridyl- 15 quinolones (U.S. Patent No. 5,330,992), styryl compounds (U.S. Patent No. 5,217,999), styryl-substituted pyridyl compounds (U.S. Patent No. 5,302,606), certain quinazoline derivatives (EP Application No. 0 566 266 A1), seleoindoles and selenides (PCT WO 94/03427, published February 17, 1994 by Denny et 20 al.), tricyclic polyhydroxylic compounds (PCT WO 92/21660, published December 10, 1992 by Dow), and benzylphosphonic acid compounds (PCT WO 91/15495, published October 17, 1991 by Dow et al).

Compounds that can traverse cell membranes and are 25 resistant to acid hydrolysis are potentially advantageous as therapeutics as they can become highly bioavailable after being administered orally to patients. However, many of these protein kinase inhibitors only weakly inhibit the function of protein kinases. In addition, many inhibit a variety of 30 protein kinases and will therefore cause multiple side-effects as therapeutics for diseases.

Some indolinone compounds, however, form classes of acid resistant and membrane permeable organic molecules. WO 96/22976 (published August 1, 1996 by Ballinari et al.) describes hydrosoluble indolinone compounds that harbor

5 tetralin, naphthalene, quinoline, and indole substituents fused to the oxindole ring. These bicyclic substituents are in turn substituted with polar moieties including hydroxylated alkyl, phosphate, and ether moieties. U.S. Patent Application Serial Nos. 08/702,232, filed August 23, 1996, entitled

10 "Indolinone Combinatorial Libraries and Related Products and Methods for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 221/187) and 08/485,323, filed June 7, 1995, entitled "Benzylidene-Z-Indoline Compounds for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 223/298)

15 and International Patent Publications WO 96/40116, published December 19, 1996 by Tang, et al., and WO 96/22976, published August 1, 1996 by Ballinari et al., all of which are incorporated herein by reference in their entirety, including any drawings, figures, or tables, describe indolinone chemical

20 libraries of indolinone compounds harboring other bicyclic moieties as well as monocyclic moieties fused to the oxindole ring. Applications 08/702,232, filed August 23, 1996, entitled "Indolinone Combinatorial Libraries and Related Products and Methods for the Treatment of Disease" by Tang et

25 al. (Lyon & Lyon Docket No. 221/187), 08/485,323, filed June 7, 1995, entitled "Benzylidene-Z-Indoline Compounds for the Treatment of Disease" by Tang et al. (Lyon & Lyon Docket No. 223/298), and WO 96/22976, published August 1, 1996 by Ballinari et al. teach methods of indolinone synthesis,

30 methods of testing the biological activity of indolinone compounds in cells, and inhibition patterns of indolinone



derivatives. Further information can be found in issued US Patent No. 5,792,783 by Tang et al. (Lyon & Lyon docket No. 223/301, filed June 5, 1996, entitled "3-Heteroaryl-2-Indolinone Compounds for the Treatment of Disease", which is  
5 also hereby incorporated herein by reference in its entirety, including any drawings, figures or tables.

Other examples of substances capable of modulating kinase activity include, but are not limited to, tyrphostins, quinazolines, quinoxolines, and quinolines. The quinazolines, tyrphostins, quinolines, and quinoxolines referred to above  
10 include well known compounds such as those described in the literature. For example, representative publications describing quinazolines include Barker et al., EPO Publication No. 0 520 722 A1; Jones et al., U.S. Patent No. 4,447,608; Kabbe et al., U.S. Patent No. 4,757,072; Kaul and Vougioukas,  
15 U.S. Patent No. 5, 316,553; Kreighbaum and Comer, U.S. Patent No. 4,343,940; Pegg and Wardleworth, EPO Publication No. 0 562 734 A1; Barker et al., (1991) Proc. of Am. Assoc. for Cancer Research 32, 327; Bertino, J.R., (1979) Cancer Research 3, 293-304; Bertino, J.R., (1979) Cancer Research 9(2 part 1), 293-304; Curtin et al., (1986) Br. J. Cancer 53, 361-368; Fernandes et al., (1983) Cancer Research 43, 1117-1123 ; Ferris et al. J. Org. Chem. 44(2), 173-178; Fry et al., (1994) Science 265, 1093-1095; Jackman et al., (1981) Cancer Research  
20 51, 5579-5586; Jones et al. J. Med. Chem. 29(6), 1114-1118; Lee and Skibo, (1987) Biochemistry 26(23), 7355-7362; Lemus et al., (1989) J. Org. Chem. 54, 3511-3518 ; Ley and Seng, (1975) Synthesis 1975, 415-522; Maxwell et al., (1991) Magnetic Resonance in Medicine 17, 189-196 ; Mini et al., (1985) Cancer  
25 Research 45, 325-330; Phillips and Castle, J. (1980) Heterocyclic Chem. 17(19), 1489-1596; Reece et al., (1977)

Cancer Research 47(11):2996-2999; Sculier et al., (1986)  
Cancer Immunol. and Immunother. 23, A65; Sikora et al., (1984)  
Cancer Letters 23, 289-295; Sikora et al., (1988) Analytical  
Biochem. 172, 344-355; all of which are incorporated herein by  
5 reference in their entirety, including any drawings.

Quinoxaline is described in Kaul and Vougioukas, U.S.  
Patent No. 5,316,553, incorporated herein by reference in its  
entirety, including any drawings.

Quinolines are described in Dolle et al., (1994) J. Med.  
10 Chem. 37, 2627-2629; MaGuire, J. (1994) Med. Chem. 37, 2129-  
2131; Burke et al., (1993) J. Med. Chem. 36, 425-432 ; and  
Burke et al. (1992) BioOrganic Med. Chem. Letters 2, 1771-  
1774, all of which are incorporated by reference in their  
entirety, including any drawings.

15 Tyrphostins are described in Allen et al., (1993) Clin.  
Exp. Immunol. 91, 141-156; Anafi et al., (1993) Blood 82:12,  
3524-3529; Baker et al., (1992) J. Cell Sci. 102, 543-555;  
Bilder et al., (1991) Amer. Physiol. Soc. pp. 6363-6143:C721-  
C730; Brunton et al., (1992) Proceedings of Amer. Assoc.  
20 Cancer Rsch. 33, 558; Bryckaert et al., (1992) Exp. Cell  
Research 199, 255-261; Dong et al., (1993) J. Leukocyte  
Biology 53, 53-60; Dong et al., (1993) J. Immunol. 151(5),  
2717-2724; Gazit et al., (1989) J. Med. Chem. 32, 2344-2352;  
Gazit et al., (1993) J. Med. Chem. 36, 3556-3564; Kaur et al.,  
25 (1994) Anti-Cancer Drugs 5, 213-222; King et al., (1991)  
Biochem.J. 275, 413-418; Kuo et al., (1993) Cancer Letters 74,  
197-202; Levitzki, A., (1992) The FASEB J. 6, 3275-3282; Lyall  
et al., (1989) J. Biol. Chem. 264, 14503-14509; Peterson et  
al., (1993) The Prostate 22, 335-345; Pillemer et al., (1992)  
30 Int. J. Cancer 50, 80-85; Posner et al., (1993) Molecular  
Pharmacology 45, 673-683; Rendu et al., (1992) Biol.

Pharmacology 44(5), 881-888; Sauro and Thomas, (1993) Life Sciences 53, 371-376; Sauro and Thomas, (1993) J. Pharm. and Experimental Therapeutics 267(3), 119-1125; Wolbring et al., (1994) J. Biol. Chem. 269(36), 22470-22472; and Yoneda et al.,  
5 (1991) Cancer Research 51, 4430-4435; all of which are incorporated herein by reference in their entirety, including any drawings.

#### VIII. Transgenic Animals

10 A variety of methods are available for the production of transgenic animals associated with this invention. DNA can be injected into the pronucleus of a fertilized egg before fusion of the male and female pronuclei, or injected into the nucleus of an embryonic cell (e.g., the nucleus of a two-cell embryo)  
15 following the initiation of cell division (Brinster et al., Proc. Nat. Acad. Sci. USA 82: 4438-4442, 1985). Embryos can be infected with viruses, especially retroviruses, modified to carry inorganic-ion receptor nucleotide sequences of the invention.

20 Pluripotent stem cells derived from the inner cell mass of the embryo and stabilized in culture can be manipulated in culture to incorporate nucleotide sequences of the invention. A transgenic animal can be produced from such cells through implantation into a blastocyst that is implanted into a foster  
25 mother and allowed to come to term. Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc.

30 The procedures for manipulation of the rodent embryo and for microinjection of DNA into the pronucleus of the zygote

are well known to those of ordinary skill in the art (Hogan et al., *supra*). Microinjection procedures for fish, amphibian eggs and birds are detailed in Houdebine and Chourrout (Experientia 47: 897-905, 1991). Other procedures for  
5 introduction of DNA into tissues of animals are described in U.S. Patent No., 4,945,050 (Sandford et al., July 30, 1990).

By way of example only, to prepare a transgenic mouse, female mice are induced to superovulate. Females are placed with males, and the mated females are sacrificed by CO<sub>2</sub>  
10 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts. Surrounding cumulus cells are removed. Pronuclear embryos are then washed and stored until the time of injection. Randomly cycling adult female mice are paired with vasectomized males. Recipient females are mated at the  
15 same time as donor females. Embryos then are transferred surgically. The procedure for generating transgenic rats is similar to that of mice (Hammer et al., Cell 63:1099-1112, 1990).

Methods for the culturing of embryonic stem (ES) cells  
20 and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection also are well known to those of ordinary skill in the art (Teratocarcinomas and Embryonic Stem Cells, A  
25 Practical Approach, E.J. Robertson, ed., IRL Press, 1987).

In cases involving random gene integration, a clone containing the sequence(s) of the invention is co-transfected with a gene encoding resistance. Alternatively, the gene encoding neomycin resistance is physically linked to the  
30 sequence(s) of the invention. Transfection and isolation of desired clones are carried out by any one of several methods

well known to those of ordinary skill in the art (E.J. Robertson, *supra*).

DNA molecules introduced into ES cells can also be integrated into the chromosome through the process of homologous recombination (Capecchi, Science 244: 1288-1292, 1989). Methods for positive selection of the recombination event (*i.e.*, neo resistance) and dual positive-negative selection (*i.e.*, neo resistance and gancyclovir resistance) and the subsequent identification of the desired clones by PCR have been described by Capecchi, *supra* and Joyner et al. (Nature 338: 153-156, 1989), the teachings of which are incorporated herein in their entirety including any drawings. The final phase of the procedure is to inject targeted ES cells into blastocysts and to transfer the blastocysts into pseudopregnant females. The resulting chimeric animals are bred and the offspring are analyzed by Southern blotting to identify individuals that carry the transgene. Procedures for the production of non-rodent mammals and other animals have been discussed by others (Houdebine and Chourrout, *supra*; Pursel et al., Science 244:1281-1288, 1989; and Simms et al., Bio/Technology 6:179-183, 1988).

Thus, the invention provides transgenic, nonhuman mammals containing a transgene encoding a Pyk2 binding polypeptide of the invention or a gene affecting the expression of the polypeptide. Such transgenic nonhuman mammals are particularly useful as an *in vivo* test system for studying the effects of introduction of a Pyk2 binding polypeptide, or regulating the expression of a Pyk2 binding polypeptide (*i.e.*, through the introduction of additional genes, antisense nucleic acids, or ribozymes).

A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell. Preferred transgenic animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats. The transgenic DNA may encode human NEK kinases. Native expression in an animal may be reduced by providing an amount of anti-sense RNA or DNA effective to reduce expression of the receptor.

#### IX. Gene Therapy

Pyk2 binding proteins or their genetic sequences will also be useful in gene therapy (reviewed in Miller, Nature 357:455-460, 1992). Miller states that advances have resulted in practical approaches to human gene therapy that have demonstrated positive initial results. The basic science of gene therapy is described in Mulligan (Science 260:926-931, 1993).

In one preferred embodiment, an expression vector containing a Pyk2 binding protein coding sequence is inserted into cells, the cells are grown *in vitro* and then infused in large numbers into patients. In another preferred embodiment, a DNA segment containing a promoter of choice (for example a strong promoter) is transferred into cells containing an endogenous gene encoding Pyk2 binding polypeptides of the invention in such a manner that the promoter segment enhances expression of the endogenous polypeptide gene (for example, the promoter segment is transferred to the cell such that it becomes directly linked to the endogenous Pyk2 binding polypeptide gene).

The gene therapy may involve the use of an adenovirus containing Pyk2 binding polypeptide cDNA targeted to a tumor, systemic Pyk2 binding polypeptide increase by implantation of engineered cells, injection with such polypeptide-encoding virus, or injection of naked Pyk2 binding polypeptide DNA into appropriate tissues.

Target cell populations may be modified by introducing altered forms of one or more components of the protein complexes in order to modulate the activity of such complexes. For example, by reducing or inhibiting a complex component activity within target cells, an abnormal signal transduction event(s) leading to a condition may be decreased, inhibited, or reversed. Deletion or missense mutants of a component, that retain the ability to interact with other components of the protein complexes but cannot function in signal transduction, may be used to inhibit an abnormal, deleterious signal transduction event.

Expression vectors derived from viruses such as retroviruses, vaccinia virus, adenovirus, adeno-associated virus, herpes viruses, several RNA viruses, or bovine papilloma virus, may be used for delivery of nucleotide sequences (e.g., cDNA) encoding recombinant Pyk2 binding polypeptides of the invention protein into the targeted cell population (e.g., tumor cells). Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors containing coding sequences (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, N.Y., 1989; Ausubel et al., Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N.Y., 1989). Alternatively, recombinant nucleic acid molecules encoding

protein sequences can be used as naked DNA or in a reconstituted system e.g., liposomes or other lipid systems for delivery to target cells (e.g., Felgner et al., Nature 337:387-8, 1989). Several other methods for the direct transfer of plasmid DNA into cells exist for use in human gene therapy and involve targeting the DNA to receptors on cells by complexing the plasmid DNA to proteins (Miller, *supra*).

In its simplest form, gene transfer can be performed by simply injecting minute amounts of DNA into the nucleus of a cell, through a process of microinjection (Capecchi, Cell 22:479-88, 1980). Once recombinant genes are introduced into a cell, they can be recognized by the cell's normal mechanisms for transcription and translation, and a gene product will be expressed. Other methods have also been attempted for introducing DNA into larger numbers of cells. These methods include: transfection, wherein DNA is precipitated with  $\text{CaPO}_4$  and taken into cells by pinocytosis (Chen et al., Mol. Cell Biol. 7:2745-52, 1987); electroporation, wherein cells are exposed to large voltage pulses to introduce holes into the membrane (Chu et al., Nucleic Acids Res. 15:1311-26, 1987); lipofection/liposome fusion, wherein DNA is packaged into lipophilic vesicles which fuse with a target cell (Felgner et al., Proc. Natl. Acad. Sci. USA. 84:7413-7417, 1987); and particle bombardment using DNA bound to small projectiles (Yang et al., Proc. Natl. Acad. Sci. 87:9568-9572, 1990). Another method for introducing DNA into cells is to couple the DNA to chemically modified proteins.

It has also been shown that adenovirus proteins are capable of destabilizing endosomes and enhancing the uptake of DNA into cells. The admixture of adenovirus to solutions containing DNA complexes, or the binding of DNA to polylysine



covalently attached to adenovirus using protein crosslinking agents substantially improves the uptake and expression of the recombinant gene (Curiel et al., Am. J. Respir. Cell. Mol. Biol. 6:247-52, 1992).

5 As used herein "gene transfer" means the process of introducing a foreign nucleic acid molecule into a cell. Gene transfer is commonly performed to enable the expression of a particular product encoded by the gene. The product may include a protein, polypeptide, anti-sense DNA or RNA, or  
10 enzymatically active RNA. Gene transfer can be performed in cultured cells or by direct administration into animals. Generally gene transfer involves the process of nucleic acid contact with a target cell by non-specific or receptor mediated interactions, uptake of nucleic acid into the cell  
15 through the membrane or by endocytosis, and release of nucleic acid into the cytoplasm from the plasma membrane or endosome. Expression may require, in addition, movement of the nucleic acid into the nucleus of the cell and binding to appropriate nuclear factors for transcription.

20 As used herein "gene therapy" is a form of gene transfer and is included within the definition of gene transfer as used herein and specifically refers to gene transfer to express a therapeutic product from a cell *in vivo* or *in vitro*. Gene transfer can be performed *ex vivo* on cells which are then  
25 transplanted into a patient, or can be performed by direct administration of the nucleic acid or nucleic acid-protein complex into the patient.

In another preferred embodiment, a vector having nucleic acid sequences encoding a Pyk2 binding polypeptide is provided  
30 in which the nucleic acid sequence is expressed only in specific tissue. Methods of achieving tissue-specific gene

expression are set forth in International Publication No. WO 93/09236, filed November 3, 1992 and published May 13, 1993.

In all of the preceding vectors set forth above, a further aspect of the invention is that the nucleic acid sequence contained in the vector may include additions, deletions or modifications to some or all of the sequence of the nucleic acid, as defined above.

In another preferred embodiment, a method of gene replacement is set forth. "Gene replacement" as used herein means supplying a nucleic acid sequence which is capable of being expressed *in vivo* in an animal and thereby providing or augmenting the function of an endogenous gene that is missing or defective in the animal.

## X. Pharmaceutical Formulations and Routes Of Administration

The substances described herein can be administered to a human patient *per se*, or in pharmaceutical compositions where it is mixed with other active ingredients, as in combination therapy, or suitable carriers or excipient(s). Techniques for formulation and administration of the substances of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition.

### a. Routes Of Administration

Suitable routes of administration may, for example, include oral, rectal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intravenous, intramedullary injections, as well

as intrathecal, direct intraventricular, intraperitoneal, intranasal, or intraocular injections.

Alternately, one may administer the compound in a local rather than systemic manner, for example, via injection of the compound directly into a solid tumor, often in a depot or sustained release formulation.

Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with tumor-specific antibody. The liposomes will be targeted to and taken up selectively by the tumor.

#### b. Composition/Formulation

The pharmaceutical compositions of the present invention may be manufactured in a manner that is itself known, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries that facilitate processing of the active substances into preparations that can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the substances can be formulated readily by combining the active substances with pharmaceutically acceptable carriers well known in the art. Such carriers enable the substances to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Suitable carriers include excipients such as, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations that can be administered orally, include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as

talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active substances may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition,  
5 stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

10 For administration by inhalation, the substances for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebuliser, with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane,  
15 dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder  
20 mix of the compound and a suitable powder base such as lactose or starch.

The substances may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be  
25 presented in unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

30 Pharmaceutical formulations for parenteral administration include aqueous solutions of the active substances in water-

soluble form. Additionally, suspensions of the active substances may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents that increase the solubility of the substances to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

The substances may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the substances may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the substances may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

A pharmaceutical carrier for hydrophobic substances is a cosolvent system comprising benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. The cosolvent system may be the VPD co-solvent system.

VPD is a solution of 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant polysorbate 80, and 65% w/v polyethylene glycol 300, made up to volume in absolute ethanol. The VPD co-solvent system (VPD:D5W) consists of VPD diluted 1:1 with a 5% dextrose in water solution. This co-solvent system dissolves hydrophobic substances well, and itself produces low toxicity upon systemic administration. Naturally, the proportions of a co-solvent system may be varied considerably without destroying its solubility and toxicity characteristics. Furthermore, the identity of the co-solvent components may be varied: for example, other low-toxicity nonpolar surfactants may be used instead of polysorbate 80; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, e.g. polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

Alternatively, other delivery systems for hydrophobic pharmaceutical substances may be employed. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophobic drugs. Certain organic solvents such as dimethylsulfoxide also may be employed, although usually at the cost of greater toxicity. Additionally, the substances may be delivered using a sustained-release system, such as semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the substances for a few weeks up to over 100 days. Depending on the chemical nature and the biological stability of the therapeutic reagent,

additional strategies for protein stabilization may be employed.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples  
5 of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

Many of the PTK modulating substances may be provided as  
10 salts with pharmaceutically compatible counterions. Pharmaceutically compatible salts may be formed with many acids, including but not limited to hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents that are  
15 the corresponding free base forms.

#### c. Effective Dosage

Pharmaceutical compositions suitable for use in the present invention include compositions where the active  
20 ingredients are contained in an amount effective to achieve its intended purpose. More specifically, a therapeutically effective amount means an amount of substance effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

25 Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any substance used in the methods of the invention, the therapeutically effective dose can be estimated initially  
30 from cell culture assays. For example, a dose can be formulated in animal models to achieve a circulating



concentration range that includes the  $IC_{50}$  as determined in cell culture (i.e., the concentration of the test compound that achieves a half-maximal inhibition of the protein Pyk2 binding polypeptide activity). Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the substances described herein can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the  $LD_{50}$  (the dose lethal to 50% of the population) and the  $ED_{50}$  (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio between  $LD_{50}$  and  $ED_{50}$ . Substances that exhibit high therapeutic indices are preferred. The data obtained from these cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage of such substances lies preferably within a range of circulating concentrations that include the  $ED_{50}$  with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration, and dosage can be chosen by the physician in view of the patient's condition. (See e.g., Fingl et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Dosage amount and interval may be adjusted individually to provide plasma levels of the active moiety which are sufficient to maintain the kinase modulating or Pyk2 binding polypeptide modulating effects, or minimal effective concentration (MEC). The MEC will vary for each compound but can be estimated from *in vitro* data; e.g., the concentration necessary to achieve 50-90% inhibition of the Pyk2 binding

polypeptide using the assays described herein. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. However, HPLC assays or bioassays can be used to determine plasma concentrations.

Dosage intervals can also be determined using MEC value. Substances should be administered using a regimen that maintains plasma levels above the MEC for 10-90% of the time, preferably between 30-90% and most preferably between 50-90%.

In cases of local administration or selective uptake, the effective local concentration of the drug may not be related to plasma concentration.

The amount of composition administered will, of course, be dependent on the subject being treated, on the subject's weight, the severity of the affliction, the manner of administration and the judgment of the prescribing physician.

#### d. Packaging

The compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accompanied with a notice associated with the container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the polynucleotide for human or veterinary administration. Such notice, for example, may be the labeling approved by the U.S. Food and Drug Administration for

prescription drugs, or the approved product insert.

Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition. Suitable conditions indicated on the label may include treatment of a tumor, inhibition of angiogenesis, treatment of fibrosis, diabetes, and the like.

#### XI. Functional Derivatives

Also provided herein are functional derivatives of a polypeptide or nucleic acid of the invention. By "functional derivative" is meant a "chemical derivative," "fragment," or "variant," of the polypeptide or nucleic acid of the invention, which terms are defined below. A functional derivative retains at least a portion of the function of the protein, for example reactivity with an antibody specific for the protein, enzymatic activity or binding activity mediated through noncatalytic domains, which permits its utility in accordance with the present invention. It is well known in the art that due to the degeneracy of the genetic code numerous different nucleic acid sequences can code for the same amino acid sequence. Equally, it is also well known in the art that conservative changes in amino acid can be made to arrive at a protein or polypeptide that retains the functionality of the original. In both cases, all permutations are intended to be covered by this disclosure.

Included within the scope of this invention are the functional equivalents of the herein-described isolated nucleic acid molecules. The degeneracy of the genetic code permits substitution of certain codons by other codons that specify the same amino acid and hence would give rise to the

same protein. The nucleic acid sequence can vary substantially since, with the exception of methionine and tryptophan, the known amino acids can be coded for by more than one codon. Thus, portions or all of the Pyk2 binding  
5 polypeptide genes could be synthesized to give a nucleic acid sequence significantly different from that shown in SEQ ID NO:3, or SEQ ID NO:4. The encoded amino acid sequence thereof would, however, be preserved.

In addition, the nucleic acid sequence may comprise a  
10 nucleotide sequence which results from the addition, deletion or substitution of at least one nucleotide to the 5'-end and/or the 3'-end of the nucleic acid formula shown in SEQ ID NO:3, or SEQ ID NO:4, or a derivative thereof. Any nucleotide or polynucleotide may be used in this regard, provided that  
15 its addition, deletion or substitution does not alter the amino acid sequence of SEQ ID NO:1, or SEQ ID NO:2 which is encoded by the nucleotide sequence. For example, the present invention is intended to include any nucleic acid sequence resulting from the addition of ATG as an initiation codon at  
20 the 5'-end of the inventive nucleic acid sequence or its derivative, or from the addition of TTA, TAG or TGA as a termination codon at the 3'-end of the inventive nucleotide sequence or its derivative. Moreover, the nucleic acid molecule of the present invention may, as necessary, have  
25 restriction endonuclease recognition sites added to its 5'-end and/or 3'-end.

Such functional alterations of a given nucleic acid sequence afford an opportunity to promote secretion and/or processing of heterologous proteins encoded by foreign nucleic  
30 acid sequences fused thereto. All variations of the nucleotide sequence of the Pyk2 binding polypeptide genes of

the invention and fragments thereof permitted by the genetic code are, therefore, included in this invention.

Further, it is possible to delete codons or to substitute one or more codons with codons other than degenerate codons to produce a structurally modified polypeptide, but one which has substantially the same utility or activity as the polypeptide produced by the unmodified nucleic acid molecule. As recognized in the art, the two polypeptides are functionally equivalent, as are the two nucleic acid molecules that give rise to their production, even though the differences between the nucleic acid molecules are not related to the degeneracy of the genetic code.

A "chemical derivative" of the complex contains additional chemical moieties not normally a part of the protein. Covalent modifications of the protein or peptides are included within the scope of this invention. Such modifications may be introduced into the molecule by reacting targeted amino acid residues of the peptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues, as described below.

Cysteiny l residues most commonly are reacted with alpha-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteiny l residues also are derivatized by reaction with bromotrifluoroacetone, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

Histidyl residues are derivatized by reaction with diethylprocarbonate at pH 5.5-7.0 because this agent is

relatively specific for the histidyl side chain. Para-bromophenacyl bromide also is useful; the reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0.

Lysinyl and amino terminal residues are reacted with  
5 succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing primary amine containing residues include  
10 imidoesters such as methyl picolinimidate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

Arginyl residues are modified by reaction with one or  
15 several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high  $pK_a$  of the guanidine functional group. Furthermore, these reagents  
20 may react with the groups of lysine as well as the arginine alpha-amino group.

Tyrosyl residues are well-known targets of modification for introduction of spectral labels by reaction with aromatic diazonium compounds or tetranitromethane. Most commonly, N-  
25 acetylimidizol and tetranitromethane are used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimide ( $R'-N-C-N-R'$ ) such as 1-cyclohexyl-3-(2-morpholinyl(4-ethyl)  
30 carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are

converted to asparaginyll and glutaminyll residues by reaction with ammonium ions.

Glutaminyll and asparaginyll residues are frequently deamidated to the corresponding glutamyl and aspartyl  
5 residues. Alternatively, these residues are deamidated under mildly acidic conditions. Either form of these residues falls within the scope of this invention.

Derivatization with bifunctional agents is useful, for example, for cross-linking the component peptides of the  
10 protein to each other or to other proteins in a complex to a water-insoluble support matrix or to other macromolecular carriers. Commonly used cross-linking agents include, for example, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-  
15 azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), and bifunctional maleimides such as bis-N-maleimido-1,8-octane. Derivatizing agents such as methyl-3-[p-azidophenyl] dithiolpropioimide yield  
20 photoactivatable intermediates that are capable of forming crosslinks in the presence of light. Alternatively, reactive water-insoluble matrices such as cyanogen bromide-activated carbohydrates and the reactive substrates described in U.S. Patent Nos. 3,969,287; 3,691,016; 4,195,128; 4,247,642;  
25 4,229,537; and 4,330,440 are employed for protein immobilization.

Other modifications include hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of  
30 lysine, arginine, and histidine side chains (Creighton, T.E., Proteins: Structure and Molecular Properties, W.H. Freeman &

Co., San Francisco, pp. 79-86 (1983)), acetylation of the N-terminal amine, and, in some instances, amidation of the C-terminal carboxyl groups.

Such derivatized moieties may improve the stability,  
5 solubility, absorption, biological half life, and the like. The moieties may alternatively eliminate or attenuate any undesirable side effect of the protein complex and the like. Moieties capable of mediating such effects are disclosed, for example, in Remington's Pharmaceutical Sciences, 18th ed.,  
10 Mack Publishing Co., Easton, PA (1990).

The term "fragment" is used to indicate a polypeptide derived from the amino acid sequence of the proteins, of the complexes having a length less than the full-length polypeptide from which it has been derived. Such a fragment  
15 may, for example, be produced by proteolytic cleavage of the full-length protein. Preferably, the fragment is obtained recombinantly by appropriately modifying the DNA sequence encoding the proteins to delete one or more amino acids at one or more sites of the C-terminus, N-terminus, and/or within the  
20 native sequence. Fragments of a protein are useful for screening for substances that act to modulate signal transduction, as described herein. It is understood that such fragments may retain one or more characterizing portions of the native complex. Examples of such retained characteristics  
25 include: catalytic activity; substrate specificity; interaction with other molecules in the intact cell; regulatory functions; or binding with an antibody specific for the native complex, or an epitope thereof.

Another functional derivative intended to be within the  
30 scope of the present invention is a "variant" polypeptide which either lacks one or more amino acids or contains



additional or substituted amino acids relative to the native polypeptide. The variant may be derived from a naturally occurring complex component by appropriately modifying the protein DNA coding sequence to add, remove, and/or to modify  
5 codons for one or more amino acids at one or more sites of the C-terminus, N-terminus, and/or within the native sequence. It is understood that such variants having added, substituted and/or additional amino acids retain one or more characterizing portions of the native protein, as described  
10 above.

A functional derivative of a protein with deleted, inserted and/or substituted amino acid residues may be prepared using standard techniques well-known to those of ordinary skill in the art. For example, the modified  
15 components of the functional derivatives may be produced using site-directed mutagenesis techniques (as exemplified by Adelman et al., 1983, DNA 2:183) wherein nucleotides in the DNA coding the sequence are modified such that a modified coding sequence is modified, and thereafter expressing this  
20 recombinant DNA in a prokaryotic or eukaryotic host cell, using techniques such as those described above.

Alternatively, proteins with amino acid deletions, insertions and/or substitutions may be conveniently prepared by direct chemical synthesis, using methods well-known in the art. The  
25 functional derivatives of the proteins typically exhibit the same qualitative biological activity as the native proteins.

Identification Of A Novel Family Of Targets Of PYK2 Related To  
*Drosophila* Retinal Degeneration B (rdgB) Protein

30 The protein tyrosine kinase PYK2 has been implicated in signaling pathways activated by G-protein-coupled receptors,

intracellular calcium, and stress signals. Here we describe the molecular cloning and characterization of a novel family of PYK2-binding proteins designated Nirs (PYK2 N-terminal domain-interacting receptors). The three Nir proteins (Nir1, Nir2, and Nir3) bind to the amino-terminal domain of PYK2 via a conserved sequence motif located in the carboxy terminus. The primary structures of Nirs reveal six putative transmembrane domains, a region homologous to phosphatidylinositol (PI) transfer protein, and an acidic domain. The Nir proteins are the human homologues of the *Drosophila* retinal degeneration B protein (rdgB), a protein implicated in the visual transduction pathways in flies. We demonstrate that Nirs are calcium-binding proteins that exhibit PI transfer activity in vivo. Activation of PYK2 by agents that elevate intracellular calcium or by phorbol ester induce tyrosine phosphorylation of Nirs. Moreover, PYK2 and Nirs exhibit similar expression patterns in several regions of the brain and retina. In addition, PYK2-Nir complexes are detected in lysates prepared from cultured cells or from brain tissues. Finally, the Nir 1-encoding gene is located at human chromosome 17p13.1, in proximity to a locus responsible for several human retinal diseases. We propose that the Nir and rdgB proteins represent a new family of evolutionarily conserved PYK2-binding proteins that play a role in the control of calcium and phosphoinositide metabolism downstream of G-protein-coupled receptors.

#### EXAMPLES

The examples below are not limiting and are merely representative of various aspects and features of the present

invention. The examples demonstrate the isolation and characterization of PYK2 binding proteins of the invention.

### Experimental Procedures

#### Sos Recruitment System

5       The Sos Recruitment System for detecting protein-protein interactions has been described by Aronheim (1997. Nucleic Acids Research 25:3373-3374; Aronheim, et al., 1997. Mol. Cell. Biol. 17:3094-3102), and includes the use of temperature-sensitive *Saccharomyces cerevisiae* strain *cdc25-2*,  
10   pADNS-h5'Sos constructs, and galactose-inducible expression library of rat pituitary cDNA fused to the Src myristylation signal.

Full-length Pyk2 was subcloned into the pADNS expression vector in frame with h5'Sos. Lysates of *S. cerevisiae cdc25-2*  
15   transfected with pADNS-h5'SOS-Pyk2 were subjected to immunoprecipitation with anti-Pyk2 antibodies followed by blotting with anti-Pyk2 or anti-Sos antibodies to verify the expression of h5'Sos-Pyk2 fusion protein.

Approximately  $4 \times 10^5$  *cdc25-2* transformants containing  
20   library plasmids and the h5'Sos-Pyk2 "bait" were grown at room temperature, replica plated onto galactose plates, and incubated at 37 °C. Library plasmids (pYES2 expression vector) were isolated from clones that exhibited galactose-dependent growth at 37 °C and retransformed into *cdc25-2* cells  
25   with pADNS vector expressing either h5'Sos-Pyk2, or non-relevant bait (h5'SOS-FAK), or h5'Sos alone. Clones which suppressed *cdc25-2* phenotype only in the presence of h5'Sos-Pyk2 were considered positive and further analyzed. Conventional yeast manipulation protocols were used. Yeast

transfection and media composition and replica plating were performed as previously described (Gietz, et al., 1996. Yeast 11:355-60; Aronheim, et al., 1997. Mol. Cell. Biol. 17:3094-3102).

## 5        Northern blot analysis

A human multiple tissues Northern blot (Clontech) was hybridized with a  $^{32}\text{P}$ -labeled cDNA fragment of Pap $\alpha$ , that corresponded to amino acids 281-691, according to manufacturer's instructions under high-stringency conditions,  
10 followed by autoradiography.

## Plasmids, subcloning, isolation of Pap cDNA, and sequence analysis

The clone obtained in the screen of the rat pituitary cDNA library, contained the C-terminal region of the Pap  
15 protein and the 3' end of the Pap gene. To identify the 5' end of the gene, a mouse brain lambda cDNA library (Stratagene) was screened with a  $^{32}\text{P}$ -labeled probe corresponding to the Pap C-terminus (639 base pairs) according to standard procedures. Positive clones were plaque-purified. Excised cDNAs were  
20 sequenced in both directions from internal and external primers using an automated sequencer (Applied Biosystems). Genetics Computer Group (GCG) sequence analysis software (University of Wisconsin, Madison) was used to analyze DNA and amino acid sequences. A Pleckstrin homology (PH) domain was  
25 identified by comparison with a PH domain database.

For mammalian expression, cDNAs encoding Pap $\alpha$  and Pap $\beta$  were subcloned into a pRK5 expression vector (Li, et al., 1992. Mol. Cell. Biol. 12:5824-33). A myc-tag was fused in

frame to the C-terminal end of Pap $\alpha$ . pRK5 vectors containing FAK, HA-tagged Pyk2, and HA-tagged PKM were also used (Lev, et al., 1995. Nature 376:737-45). To generate PC12 cells stably expressing Pap $\beta$ , cDNA of Pap $\beta$  was subcloned into a pLXSN  
5 retrovirus using PCR.

To create the GST-PAP fusion protein, the C-terminal part of Pap, encoding the proline-rich region and the SH3 domain was amplified by PCR and subcloned into pGEX-2T vector (Pharmacia Biotech, Inc.) in frame with GST. The construct  
10 was expressed in *Escherichia coli*, and purified by affinity chromatography on glutathione-Sepharose beads (Smith, et al., 1988. Gene 67:31-40).

To create the construct to be used in the Arf GAP assay, the region of Pap $\beta$  containing the PH domain, the Arf GAP  
15 domain, and the ankyrin homology region (amino acids 111-522), was amplified by PCR and subcloned into the bacterial expression vector pET22b(+) (Novagen) in frame with the His-tag sequence. The protein product was expressed in bacteria and purified by sequential chromatography on Hiload Q and  
20 Nickel-chelating columns (Pharmacia Biotech, Inc.).

#### Cell lines, transient and stable transfections

Human kidney (293), monkey kidney (COS-7), and human epithelial (HeLa) cells were grown in DMEM (Cellgro) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). Rat pheochromocytoma (PC12) cells were grown in DMEM  
25 with 10% fetal bovine serum and 10% horse serum. PC12 cells were starved in growth medium without serum for 24 h before stimulation with PMA (2  $\mu$ M) for 10 min, or with the mixture of H<sub>2</sub>O<sub>2</sub> and NaVO<sub>3</sub> (1 mM) for 20 min. Nearly confluent 293 cells

were transfected with 1  $\mu$ g DNA per well of a 6-well plate using the calcium precipitation method (Chen, et al., 1987. Mol. Cell. Biol. 7:2745-52), or as indicated. Stably transfected PC12 cells expressing Pap $\beta$  were generated by using the PLXSN retroviral expression vector (Hadari, et al., 1998. Mol. Cell. Biol. 18:3966-3973). The expression level of Pap $\beta$  was determined by immunoblotting PC12 cell lysates after selection.

#### Antibodies, Immunoprecipitation and Immunoblotting

Antibodies against Pap were raised in rabbits immunized with KLH-conjugated synthetic peptides corresponding to amino acids 612-623 (Pap $\beta$ ) or with the GST-PAP fusion protein (see above). Antibodies against FAK were obtained from Transduction Laboratories. Antibodies against Pyk2 were previously described (Dikic, et al., 1996. Nature 383:547-550). Anti-Pyk2 antibodies recognized Pyk2 and PKM, but did not recognize FAK or Pap. Polyclonal anti-phosphotyrosine antibodies were previously described (Batzner, et al., 1994. Mol. Cell. Biol. 14:5192-201). The anti-mannosidase II and anti- $\beta$ -COP polyclonal antibodies were provided by Academic Laboratories. Rabbit polyclonal anti-Sos, anti-Src antibodies, anti-HA tag, anti-myc tag, and anti-GST monoclonal antibodies were from Santa Cruz Biotechnology, Inc.

For immunoprecipitation and immunoblotting analysis, the cells were washed with ice-cold phosphate-buffered saline and lysed in 50 mM Hepes, pH 7.2, 150 mM NaCl, 1 mM EDTA, 10% Glycerol, 1% Triton X-100, 1 mM Sodium Orthovanadate, 40 mM  $\beta$ -Glycerophosphate, 10 mM Sodium Pyrophosphate, 1 mM PMSF, 10  $\mu$ g/mL Leupeptin, 10  $\mu$ g/mL Aprotinin (lysis buffer). Cell

extracts were precleared by centrifugation and loaded on SDS-PAGE (total lysate), or incubated with antibodies cross-linked to Protein A-Sepharose beads in a nutator at 4 °C for 3h, or overnight. Immunocomplexes were washed in lysis buffer and  
5 analyzed as described (Galisteo, et al., 1996. J. Biol. Chem. 271:20997-1000).

For Far-Western blots, total cell lysates and immunoprecipitates from transfected 293 cells were separated on SDS-PAGE and transferred to nitrocellulose using  
10 conventional techniques. Filters were blocked overnight with TBS buffer containing 5% BSA at 4 °C, and incubated with a mixture of GST-PAP fusion protein (3 µg/mL) and anti-GST monoclonal antibodies (1:1000) overnight at 4 °C. Filters were then processed as regular immunoblots.

15 Mouse brain homogenate (20% w/v) was prepared by rapidly excising the brain from sacrificed mice, briefly soaking the tissue in ice-cold phosphate-buffered saline, followed by homogenizing the tissue using a Teflon-glass homogenizer (ten strokes) in ice-cold lysis buffer. The extract was then  
20 centrifuged at 4 °C in a table-top centrifuge for 10 min at maximum speed, and then recentrifuged at 4 °C for 1h (100,000 x g). The supernatant was used for immunoprecipitation and immunoblotting.

#### Arf GAP assay

25 Arf GAP activity was determined using an *in vitro* assay that measures a single round of GTP hydrolysis on recombinant Arf (Randazzo, et al., 1994. J. Biol. Chem. 269:10758-63). Crude phosphoinositides, phosphatidylinositol (PI) from bovine liver, phosphatidylinositol 4,5-biphosphate (PIP<sub>2</sub>),

phosphatidylcholine (PC), phosphatidylserine (PS) from bovine brain, and phosphatidic acid (PA) prepared from lecithin were obtained from Sigma. Phospholipids were solubilized in 0.1% Triton X-100 and were added to the assay as mixed micelles.

5        Myristoylated Arf1, nonmyristoylated Arf1, Arl2 and myristoylated Arf5 were prepared as described (Randazzo, et al., 1992. Methods Enzymol. 219:362-369; Randazzo, 1997. Biochem J. 324:413-9). To compare the Arf isoform specificity of Pap, all Arfs were used in myristoylated form. The cDNA  
10        for Arf6 was expressed in *Escherichia coli* strain BL21(DE3) and was purified as described (Brown, et al. Src. Mol. Cell. Biol.). To determine Arf GAP activity in cell lysates, 293 cells extracts were prepared as described above.

#### Immunofluorescence analysis and subcellular fractionation

15        HeLa or COS-7 cells were grown on uncoated coverslips, transfected using the calcium precipitation method, washed twice in PBS (37 °C), fixed in 2% formaldehyde for 20 min at room temperature, permeabilized in PBS containing Triton X-100 (0.2%) for 20 min, and washed with PBS. Coverslips were  
20        incubated with primary antibodies or preimmune serum diluted in TBS buffer containing BSA (5%) for one hour, washed in PBS, incubated with secondary antibodies for one hour, washed in PBS, mounted with fluorostab (ICN Pharmaceuticals, Inc.), and inspected with a confocal microscope (Sarastro-2000). For  
25        double label immunofluorescence experiments the incubation with primary (rabbit) and secondary (anti-rabbit) antibodies was followed by incubation with the second set of primary (murine) and secondary (anti-mouse) antibodies. In control experiments primary antibodies from the second set were not



used. For subcellular fractionation, 293 cells overexpressing Pap $\alpha$  were lysed in lysis buffer without detergent by repeatedly freezing and thawing three times. Total lysate (T) was separated into soluble (S) and particulate (P) fractions  
5 by a 30 min centrifugation at 16,000 x g at 4 °C.

#### In vitro generation of post-Golgi vesicles

Golgi fractions were isolated from VSV-infected MDCK cells and the cytosolic proteins fractionated from rat liver cytosol (Simon, et al., 1996. J. Biol. Chem. 271:16952-61).  
10 The Golgi fractions contained <sup>35</sup>S labeled sialylated VSV-G protein that had been allowed to accumulate in the Trans-Golgi Network (TGN) during incubation of the cells at 20 °C for 2h prior to lysis. Vesicle generation proceeded during the incubation at 37 °C for 60 min and was supported by either ATP  
15 (1 mM) or the poorly hydrolyzable GTP analog guanylyl-imidodiphosphate (GMP-PNP) (100  $\mu$ M). The reactions were terminated by cooling on ice. Golgi membranes were removed by sedimentation at 10,000 x g for 10 min. The release of labeled viral glycoprotein was measured as the percentage of  
20 total labeled protein initially present in the Golgi fraction that appeared in the supernatant. In some instances cooled reactions were analyzed by sucrose density gradient centrifugation which allowed separation of the released vesicles (Simon, et al., 1996. J. Biol. Chem. 271:16952-61).

#### 25 Secreted Alkaline Phosphatase (SEAP) assay

Human 293 cells grown in six-well plates were co-transfected with pCDNA.3SEAP and pRK5-based constructs (1:100) using the calcium precipitation method. Thirty-six hours

after transfection, the medium was changed. Four hours later the culture supernatants were removed, and the cells were lysed in ice-cold growth medium supplemented with 1% Triton X-100 and 1 mM PMSF, 10 µg/mL Leupeptin, 10 µg/mL Aprotinin.

5 SEAP activity in the culture supernatants and in the cell lysates was determined using the SEAP Reporter Gene Assay (Boehringer, Mannheim). The presence of 1% Triton X-100 did not affect the SEAP activity in cell lysates (not shown). Secretion was expressed as the ratio of SEAP activity in  
10 culture supernatants to the sum of SEAP activity in culture supernatants and cell lysates. All experiments were performed three times in triplicate. The expression of pRK5-based constructs was verified by immunoblots.

15 EXAMPLE 1: ISOLATION OF A PYK2 ASSOCIATED PROTEIN USING THE SOS RECRUITMENT SYSTEM

The Sos Recruitment System (SRS) (also known as the cytoplasmic two-hybrid screen) is a genetic method that  
20 enables detection of protein-protein interactions in yeast (Aronheim, et al., 1997. Mol. Cell. Biol. 17:3094-3102). SRS was used to screen a rat pituitary cDNA library with Pyk2 as a bait to Identify Pyk2 binding proteins.. One clone that interacted specifically with Pyk2, but not with a heterologous  
25 bait (FAK), was isolated after screening  $4 \times 10^5$  transformants. This clone (639 bases) was used to identify two new clones by screening a mouse brain cDNA library.

Pap $\alpha$  and Pap $\beta$  cDNAs were transiently expressed in 293 cells. Proteins with the appropriate molecular weight were  
30 detected by immunoprecipitation with polyclonal rabbit anti-Pap antibodies followed by SDS PAGE and autoradiography.

The tissue expression pattern of Pap was determined by Northern blot analysis of various human tissues with a specific Pap probe (Fig. 3). A Pap transcript of approximately 5.7 kb was detected predominantly in brain, kidney, and heart, as well as in placenta, lungs and pancreas.

EXAMPLE 2: IN VITRO AND IN VIVO ASSOCIATION BETWEEN PYK2 AND PAP

The association between Pap and Pyk2 was confirmed by Far-Western blot analysis. A GST fusion protein containing the proline-rich region and the SH3 domain of Pap was used as a probe for examining direct binding to Pyk2. This region of Pap binds specifically to Pyk2 and to the kinase-negative Pyk2 mutant-PKM (Fig. 4a).

We next co-transfected 293 cells with expression vectors for Pyk2-HA and Pap $\beta$ , for PKM-HA and Pap $\beta$ , For each separately. The transfected cells were lysed, subjected to immunoprecipitation and immunoblotting with anti-Pap and anti-HA antibodies. Pap $\beta$  forms a complex with Pyk2 and with PKM, indicating that the association between these two proteins is independent of the kinase activity of Pyk2 (Fig. 4b).

Association between Pyk2 and Pap was also detected in lysates prepared from mouse brain and from PC12 cells infected with Pap $\beta$  virus (Fig. 4c). In brain lysates, anti-Pyk2 antibodies immunoprecipitated a protein that migrated in SDS with an apparent molecular weight of 112 kDa, that was specifically recognized by anti-Pap antibodies (Fig. 4c, upper right panel). Similarly, in PC12 cells infected with Pap $\beta$  virus, anti-Pyk2 antibodies immunoprecipitated both

exogenously expressed murine 90 kDa form of Pap $\beta$  and the endogenous 112 kDa form of rat Pap $\alpha$  (Fig. 4c, upper left panel).

The proline rich region of Pap $\alpha$  and pap $\beta$  contains a PPLPPRNVGK sequence that closely resembles the consensus binding site for the SH3 domain of Src (Rickles, et al., 1995. Proc. Natl. Acad. Sci. USA 92:10909-13). Human 293 cells were transfected with expression vectors for Src and Pap $\alpha$ , and lysates from transfected cells were subjected to immunoprecipitation with anti-Pap antibodies followed by immunoblotting with anti-Src antibodies. Stable complex formation between Pap $\alpha$  and Src was found in lysates from these cells (Fig. 4d).

15 EXAMPLE 3: TYROSINE PHOSPHORYLATION OF PAP BY PYK2 OR SRC  
KINASES

Both phorbol ester (PMA) and pervanadate (NaVO<sub>3</sub>) stimulate tyrosine phosphorylation of Pyk2 in PC12 and other cell types (Dikic, et al., 1996. Nature 383:547-550; Lev, et al., 1995. Nature 376:737-45). PC12 cells, stimulated or unstimulated with pervanadate or PMA, were lysed and subjected to immunoprecipitation with anti-Pap antibodies, followed by immunoblotting with antibodies against phosphotyrosine. Both PMA and vanadate treatment induce tyrosine phosphorylation of Pap $\alpha$  (Fig. 5a).

To study Pap phosphorylation by PYK2, 293 cells were co-transfected with expression vectors for Pyk2-HA and Pap $\beta$ , or for PKM-HA and Pap $\beta$ . Cell lysates were subjected to immunoprecipitation with anti-Pap antibodies, and

phosphorylation of Pap $\beta$  on tyrosine residues was determined by immunoblotting with anti-phosphotyrosine antibodies (Fig. 5b). Analysis of Pap $\beta$  immunoprecipitates of lysates prepared from cells co-expressing Pap $\beta$  and Pyk2, demonstrated that the two  
5 proteins form a complex and are tyrosine phosphorylated. By contrast, Pap $\beta$  was not tyrosine phosphorylated in cells expressing Pap $\beta$  alone or in cells co-expressing Pap $\beta$  and the kinase negative mutant of Pyk2-PKM (Fig. 5b).

To examine the specificity of the Pyk2 and Pap  
10 interaction, 293 cells were co-transfected with Pap $\beta$  and Pyk2, or with Pap $\beta$  and FAK. Pap $\beta$  is tyrosine phosphorylated in Pyk2 expressing cells, but not in FAK expressing cells, (Fig. 6). Trace amounts of FAK were found in PAP $\beta$  immunoprecipitates following extreme overexpression of FAK. However, no tyrosine  
15 phosphorylation of PAP $\beta$  was detected. Taken together, these experiments demonstrate that Pap $\beta$  is tyrosine phosphorylated by Pyk2, but not by the closely related kinase FAK.

To determine whether Pap is also phosphorylated by Src, lysates from 293 cells co-transfected with Pap $\alpha$  and Src were  
20 separated on SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies. The same filter was reprobed with anti-Src and anti-PAP antibodies. Src and Pap $\alpha$  are tyrosine phosphorylated when co-expressed in 293 cells (Fig. 5c). Tyrosine phosphorylation of Pap $\alpha$  was not detected in lysates  
25 from 293 cells co-transfected with expression vectors for Pap $\alpha$  and for a kinase negative mutant of Src (Fig. 5c, right panel).

EXAMPLE 4: PAP PROTEIN EXHIBITS ARF GAP ACTIVITY IN VITRO

The presence of an Arf GAP domain in the Pap amino acid sequence (Fig. 2) implies that Pap may activate GTP hydrolysis by Arf. The part of Pap containing the PH domain, the Arf GAP domain, and the ankyrin homology region (amino acids 111-522), was expressed in bacteria and tested *in vitro* for Arf GAP activity (Randazzo, et al., 1994. J. Biol. Chem. 269:10758-63) using Arf1, Arf5, Arf6 and Arl2 as substrates. In the presence of crude phosphoinositides containing mainly PtdIns(4,5)P<sub>2</sub>, recombinant Pap exhibited GAP activity towards Arf1 and Arf5, weaker activity towards Arf6, and no activity towards Arl2 (Fig. 7a). GAP activity was detected only in the presence of PtdIns(4,5)P<sub>2</sub>, not in the presence of phosphatidic acid (PA), phosphatidylinositol (PI) phosphatidyl serine (PS), or phosphatidylcholine (PC) (Fig. 7b). Lysates prepared from 293 cells transfected with myc-tagged Pap $\alpha$  had approximately 100 fold greater Arf GAP activity towards Arf1 as compared to lysates prepared from cells transfected with vector alone (not shown).

EXAMPLE 5: INTRACELLULAR LOCALIZATION OF PAP

The intracellular localization of Pap was determined using immunofluorescence microscopy. Inspection of permeabilized, fluorescently labeled HeLa cells overexpressing myc-tagged Pap $\alpha$  showed that Pap $\alpha$  is located in the cytoplasm and at the edge of the cells in membrane protrusions (Fig. 9). Inspection of permeabilized HeLa or COS-7 cells overexpressing Pap $\alpha$  and Pyk2-HA using double label

immunofluorescence microscopy with anti-Pap and anti-HA antibodies, respectively, demonstrated that in both cell lines Pyk2 and Pap $\alpha$  are localized in the plasma membrane and in membrane protrusions (Fig. 10). These results are consistent with a subcellular fractionation experiment demonstrating that a certain amount of overexpressed PAP $\alpha$  protein is constantly associated with the particulate fraction (Fig. 8).

The cytoplasmic location of Pap was further analyzed by performing double label immunofluorescence microscopy analysis with antibodies that recognize known marker proteins. In these experiments, COS-7 cells overexpressing Pap $\alpha$ -myc were permeabilized and were labeled with anti-myc antibodies and with antibodies that recognize specific intracellular compartments. These experiments demonstrated that a population of Pap $\alpha$  molecules is co-localized with  $\beta$ -Cop and Mannosidase II; two specific markers of the Golgi compartment (Fig. 11). Overexpression of Pap $\alpha$  did not influence the integrity of the Golgi compartment. In contrast, overexpression of Arf1-GAP causes fusion of the Golgi complex with the endoplasmic reticulum (ER) (Aoe, et al., 1997. EMBOJ. 16:7305-16).

EXAMPLE 6: ENHANCING PAP LEVELS INHIBITS GENERATION OF POST-GOLGI VESICLES AND REDUCES CONSTITUTIVE SECRETION

The role of Arf1-GTP in vesicle budding from the Trans-Golgi Network (TGN) is well established (Donaldson, et al., 1994. Curr. Opin. Cell. Biol. 6:527-32; Simon, et al., 1996. J. Biol. Chem. 271:16952-61). Immunofluorescence localization experiments described herein suggest that one of the potential

sites of PAP action is in the Golgi compartment. Recruitment of Pap to the Golgi compartment may inhibit vesicle budding by reducing the pool of Arf1-GTP associated with TGN.

An *in vitro* system for the generation of post-Golgi vesicles from an isolated Golgi fraction prepared from vesicular stomatitis virus (VSV) infected MDCK cells (Simon, et al., 1996. J. Biol. Chem. 271:16952-61) was used to test this hypothesis. In this system, vesicle generation is cytosol and temperature dependent, and requires a source of nucleotide triphosphates. In the presence of ATP, the released vesicles are 50 to 80 nm in diameter and lack coat structure (Simon, et al., 1996. J. Biol. Chem. 271:16952-61). When ATP is replaced by the poorly hydrolyzable GTP analog, guanylyl-imidodiphosphate (GMP-PNP), the released post-Golgi vesicles remain coated with a non-clathrin COP-1 coat due to the fact that uncoating requires hydrolysis of GTP bound to Arf (Simon, et al., 1996. J. Biol. Chem. 271:16952-61).

In the presence of ATP, recombinant Pap inhibits vesicle generation in a concentration dependent manner (Fig. 12a & 12b). When nucleotides were excluded from the reaction mixture, no vesicle production occurred (Fig. 12C). When GMP-PNP was used instead of ATP, Pap did not have any effect on coated vesicle production (Fig. 12D). The fact that PAP could only inhibit vesicle release in the presence of hydrolysable nucleotides indicates that it acts as an Arf GAP that prevents the stable association of Arf with the TGN membranes.

The effect of Pap overexpression on secretion *in vivo* was tested using the SEAP Assay. When expressed in transfected cells, the truncated form of placental alkaline phosphatase (SEAP) serves as a marker to assess constitutive secretion (Gorr, 1996. J. Biol. Chem. 271:3575-3580). This protein



undergoes N-glycosylation in the Golgi apparatus from which it is transported to the plasma membrane for release into the culture medium.

Approximately 80% of total SEAP synthesized in  
5 transiently transfected 293 cells was released in the medium after 4 hours (Fig. 13). As expected, Brefeldin A treatment blocked secretion completely, indicating that this process requires Arf-GTP. However, when Pap was co-transfected with SEAP into 293 cells, a small but reproducible decrease in SEAP  
10 secretion was detected (Fig. 13). Co-transfection with Pyk2 did not affect either SEAP secretion or Pap-mediated inhibition of SEAP secretion.

One skilled in the art would readily appreciate that the present invention is well adapted to carry out the objects and  
15 obtain the ends and advantages mentioned, as well as those inherent therein. The molecular complexes and the methods, procedures, treatments, molecules, specific compounds described herein are presently representative of preferred embodiments are exemplary and are not intended as limitations  
20 on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention are defined by the scope of the claims.

It will be readily apparent to one skilled in the art  
25 that varying substitutions and modifications may be made to the invention disclosed herein without departing from the scope and spirit of the invention.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in  
30 the art to which the invention pertains.

The invention illustratively described herein suitably may be practiced in the absence of any element or elements, limitation or limitations which is not specifically disclosed herein. Thus, for example, in each instance herein any of the  
5 terms "comprising", "consisting essentially of" and "consisting of" may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and there is no intention that in the use of such terms and expressions  
10 of excluding any equivalents of the features shown and described or portions thereof, but it is recognized that various modifications are possible within the scope of the invention claimed.

In addition, where features or aspects of the invention  
15 are described in terms of Markush groups, those skilled in the art will recognize that the invention is also thereby described in terms of any individual member or subgroup of members of the Markush group. For example, if X is described as selected from the group consisting of bromine, chlorine,  
20 and iodine, claims for X being bromine and claims for X being bromine and chlorine are fully described.

Finally, the invention has been described broadly and generically herein. Each of the narrower species and subgeneric groupings falling within the generic disclosure  
25 also form part of the invention. This includes the generic description of the invention with a proviso or negative limitation removing any subject matter from the genus, regardless of whether or not the excised material is specifically recited herein.

30 Other embodiments are within the following claims.

What is claimed is:

CLAIMS

1. An isolated, enriched or purified nucleic acid  
5 molecule encoding a Pyk2 binding protein.

2. The nucleic acid molecule of claim 1, wherein said  
Pyk2 binding protein is selected from the group consisting of  
Pap $\alpha$  and Pap $\beta$ .

10

3. The nucleic acid molecule of claim 1, wherein said  
nucleic acid molecule comprises a nucleotide sequence that:

(a) encodes a polypeptide having the amino acid  
sequence set forth in SEQ ID NO:1 or SEQ ID NO:2;

15

(b) is the complement of the nucleotide sequence of  
(a);

(c) hybridizes under stringent conditions to the  
nucleotide molecule of (a) and encodes a naturally occurring  
Pyk2 binding protein;

20

(d) encodes a polypeptide having the amino acid  
sequence set forth in SEQ ID NO:1 or SEQ ID NO:2, except that  
it lacks one or more, but not all, of the domains selected  
from the group consisting of N-terminal, coiled-coil, PH, Arf  
GAP, Ankyrin, proline rich, SH3, and C-terminal domains; or

25

(e) is the complement of the nucleotide sequence of  
(d).

4. The nucleic acid molecule of claim 1, further  
comprising a vector or promoter effective to initiate  
30 transcription in a host cell.

5. The nucleic acid molecule of claim 1, wherein said nucleic acid molecule is isolated, enriched, or purified from a mammal.

5 6. The nucleic acid molecule of claim 5, wherein said mammal is a human.

7. A nucleic acid probe for the detection of nucleic acid encoding a Pyk2 binding protein in a sample.

10

8. The probe of claim 7, wherein said protein is selected from the group consisting of Pap $\alpha$  and Pap $\beta$ .

9. The probe of claim 7, wherein said protein is a  
15 fragment of the protein encoded by the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2.

10. A recombinant cell comprising a nucleic acid molecule encoding a Pyk2 binding protein.

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11. The cell of claim 10, wherein said protein is selected from the group consisting of Pap $\alpha$  and Pap $\beta$ .

12. The cell of claim 10, wherein said protein is a  
25 fragment of the protein encoded by the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2.

13. An isolated, enriched, or purified Pyk2 binding protein.

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14. The protein of claim 13, wherein said protein is selected from the group consisting of Pap $\alpha$  and Pap $\beta$ .

15. The protein of claim 13, wherein said protein is a fragment of the protein encoded by the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2.

16. The protein of claim 13, wherein said protein comprises an amino acid sequence having

10 (a) the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2; or

(b) the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2, except that it lacks one or more, but not all, of the domains selected from the group consisting of N-terminal, catalytic, C-terminal, coiled-coil, PH, Arf GAP, 15 Ankyrin repeat, proline rich, and SH3 domains.

17. The Pyk2 binding protein of claim 13, wherein said protein is isolated, purified, or enriched from a mammal.

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18. The Pyk2 binding protein of claim 17, wherein said mammal is a human.

19. An antibody or antibody fragment having specific 25 binding affinity to a Pyk2 binding protein or to a domain of said protein.

20. The antibody or antibody fragment of claim 19, wherein said protein is selected from the group consisting of 30 Pap $\alpha$  and Pap $\beta$ .

21. A hybridoma which produces an antibody having specific binding affinity to a Pyk2 binding protein.

22. The hybridoma of claim 21, wherein said protein is  
5 selected from the group consisting of Pap $\alpha$  and Pap $\beta$ .

23. A method for identifying a substance that modulates Pyk2 binding protein activity comprising:

- 10 (a) contacting a Pyk2 binding protein with a test substance;  
(b) measuring the activity of said protein; and  
(c) determining whether said substance modulates the activity of said protein.

15 24. The method of claim 23, further comprising the addition of a natural binding partner to part (a).

20 25. The method of claim 24, wherein said natural binding partner is selected from the group consisting of Pyk2 and Src.

26. A method for identifying a substance that modulates Pyk2 binding protein activity in a cell comprising:

- 25 (a) expressing Pyk2 binding protein in a cell;  
(b) providing a test substance to said cell; and  
(c) monitoring a change in cell phenotype or the interaction between said protein and a natural binding partner to identify said substance that modulates Pyk2 binding partner activity in a cell.

30 27. The method of claim 26, wherein said natural binding partner is selected from the group consisting of Pyk2 and Src.

28. A method for treating a disease or a disorder by administering to a patient in need of such treatment a substance that modulates the activity of a Pyk2 binding protein.

5

29. The method of claim 28, wherein said disease or disorder is selected from the group consisting of cancer, cardiovascular, neurodegenerative, and immune disorders.

10

30. The method of claim 28, wherein said substance modulates Pyk2 binding protein activity *in vitro*.

31. The method of claim 28, wherein said substance inhibits Pyk2 binding protein activity.

15

32. The method of claim 31, wherein said substance is an antisense oligonucleotide that inhibits the expression of Pyk2 binding protein.

20

33. A method for detection of nucleic acid encoding a Pyk2 binding protein in a sample as a diagnostic tool for a disease or a disorder, wherein said method comprises:

(a) contacting said sample with a nucleic acid probe which hybridizes under hybridization assay conditions to a nucleic acid target region of said protein, said probe comprising the nucleic acid sequence encoding said protein, fragments thereof, and the complements of said sequences and fragments; and

25

(b) detecting the presence or amount of the probe:target region hybrid as an indication of said disease.

30

34. The method of claim 33, wherein said disease is selected from the group consisting of cancer, cardiovascular, neurodegenerative, and immune disorders.

5 35. An antisense oligonucleotide that inhibits the expression of Pyk2 binding protein.

36. The antisense oligonucleotide of claim 35, wherein said oligonucleotide is the complement of a sequence encoding  
10 a fragment of the protein encoded by the amino acid sequence set forth in SEQ ID NO:1 or SEQ ID NO:2.

15

20

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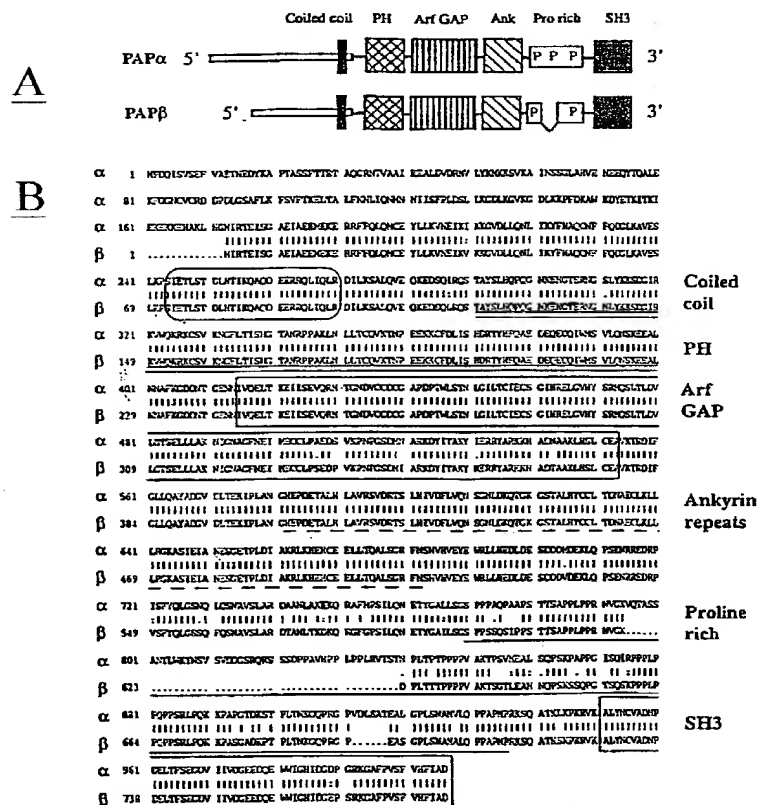
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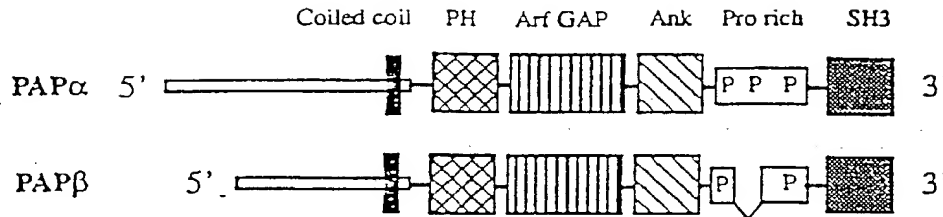
(54) Title: PYK2 BINDING PROTEINS



[Continued on next page]

A

FIGURE 1

B

α	1	KFDQISVSEF	VAETHEDYKA	PTASSFTTST	AOCRNTVAAI	EEALDVRNV	LYGCKSVKA	INSSGLAWE	KEDYQTALE
α	81	KFGNCVCRD	DPELGSAPLK	FSVFTXELTA	LFNRLIQNN	NIISFPLDSL	LKGLKGVKG	DLKXPFDAW	KDYETKITKI
α	161	EKEGCHAKL	HCHIRTEISG	AETAEDEKE	RRFQLOHCE	YLLKVAEIKI	KKGVDLLQNL	IKYFHAQNF	FQDGLKAVES
β	1	.....	HIRTEISG	AETAEDEKE	RRFQLOHCE	YLLKVAEIKV	KKGVDLLQNL	IKYFHAQNF	FQDGLKAVES
α	241	LQSIETLST	DLHTIKQAO	EERRQLIQLR	DILKSALQVE	QKEDSQIRCS	TAYSLHQPCG	NKCHTERNG	SLYKSDGIR
β	69	LQSIETLST	DLHTIKQAO	EERRQLIQLR	DILKSALQVE	QKEDQLRCS	TAYSLHQPCG	NKCHTERNG	SLYKSDGIR
α	321	KWQKRCQSV	KNGFLTISG	TANRPPAKLN	LUTCQVKNP	EEKCFDLIS	HDRTYHQAE	DEECQIAMS	VLQSKSEAL
β	149	KWQKRCQSV	KNGFLTISG	TANRPPAKLN	LUTCQVKNP	EEKCFDLIS	HDRTYHQAE	DEECQIAMS	VLQSKSEAL
α	401	NAFKGDDNT	GENIVQELT	KEIISEVQNH	TQNDVCCDCG	APDPTWLSTN	LGILTCIECS	GIHRELGVHY	SRQSLTLOV
β	229	NAFKGDDNT	GENIVQELT	KEIISEVQNH	TQNDVCCDCG	APDPTWLSTN	LGILTCIECS	GIHRELGVHY	SRQSLTLOV
α	481	LQSELLAK	NIGHAGFNEI	HECCLPASDS	VKNFGSDNI	ARKDYITAKY	HERRYAPKNI	ADNAAXLHSL	CEVXTROIF
β	309	LQSELLAK	NIGHAGFNEI	HECCLPSEDP	VKNFGSENI	ARKDYITAKY	HERRYAPKNI	ADTAAXLHSL	CEVXTROIF
α	561	GLLQYADGV	DLTEKIPLAN	CHEPDOTALH	LAVRSVORTS	LHIVDFLVCH	SCHLEKQTK	GSTALHYCC	TAIAECLALL
β	384	GLLQYADGV	DLTEKIPLAN	CHEPDOTALH	LAVRSVORTS	LHIVDFLVCH	SCHLEKQTK	GSTALHYCC	TAIAECLALL
α	641	LPGKASIEIA	NESGETPLDI	AKRLKHEHCE	ELLTQALSCR	FNSHVHVEYE	WRLHEDLDE	SQOVDKXLQ	PSGNRRDRP
β	469	LPGKASIEIA	NESGETPLDI	AKRLKHEHCE	ELLTQALSCR	FNSHVHVEYE	WRLHEDLDE	SQOVDKXLQ	PSGNRRDRP
α	721	ISFYQLGSSQ	PGSNVSLAR	DAANLAKENQ	RAFHPISLQN	ETYGALLSSS	PPHPAPAPS	TTSAPPLPPR	NVGVQVASS
β	549	VSFYQLGSSQ	PGSNVSLAR	DTANLTNDKQ	RGFPSISLQN	ETYGALLSSS	FPSSQSIPPS	TTSAPPLPPR	NVGVQVASS
α	801	ANTLAKTNSV	SVEGCSQRQS	SSOPPAVHPP	LPPLRVSTNI	PLTPTPPPPV	AKTPSVNEAL	SQPSKPAPPG	ISQIRPPPLP
β	623	.....	.....	.....	.....	PLTPTPPPPV	AKTGTLEAH	KQPSKSSQPG	TSCSKPPPLP
α	881	FQPPSRLPKQ	KPAGTQKST	PLTNQOQPRG	PVOLSATEAL	GPLSNAMVLO	PPAPHPKRSQ	ATKLKPKRVK	ALYNCAVNDP
β	664	FQPPSRLPKQ	KPAGTQKPT	PLTNQOQPRG	P.....EAS	GPLSNAMVLO	PPAPHPKRSQ	ATKLKPKRVK	ALYNCAVNDP
α	961	DELTFSEGV	IIVGEEEDCE	WHIGHIDGP	GRKGFVPSF	VHFIAD	.....	.....	.....
β	738	DELTFSEGV	IIVGEEEDCE	WHIGHIDGP	SRKGFVPSF	VHFIAD	.....	.....	.....

Coiled  
coil

PH

Arf  
GAPAnkyrin  
repeatsProline  
rich

SH3

FIGURE 2

ARF1 GAP	1	N	A	S	R	K	V	K	R	A	Q	D	E	N	F	E	L	F	V	V	T					
GCS	1	K	D	D	R	R	L	O	L	K	I	G	A	K	K	M	A	T	P	K						
PAP $\beta$	1	I	Q	E	L	K	E	I	S	R	M	T	G	D	C	D	T	L	T	N						
ARF1 GAP	37	Y	W	R	L	V	K	W	D	I																
GCS	37	F	A	F	A	I	Q	F	P	E																
PAP $\beta$	37	L	L	T	I	E	Y	R	M	Q	L	L	V	L	G	T	S									
ARF1 GAP	73	E	K	A	K	R	F	L	-	-	-	A	Q	D	Y	E	-	-	-	W	S	L				
GCS	73	R	E	K	E	P	L	T	W	F	K	-	-	-	H	N	I	D	L	-	-	L	P	Q		
PAP $\beta$	73	L	A	N	I	G	N	I	M	C	C	L	P	E	P	V	K	N	P	G	S	D	M	I	A	R
ARF1 GAP	100	O	-	-	-	S	S	-	-	A	L	F	-	-	-	R	D	V	A	T	A	G				
GCS	99	V	-	-	-	D	N	P	-	V	E	D	Y	-	-	K	E	T	C	D						
PAP $\beta$	109	Y	I	T	A	M	E	R	Y	R	K	K	H	A	D	T	A	A	H	S	A					

FIGURE 3

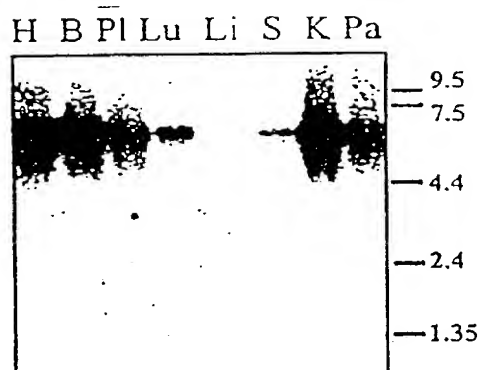


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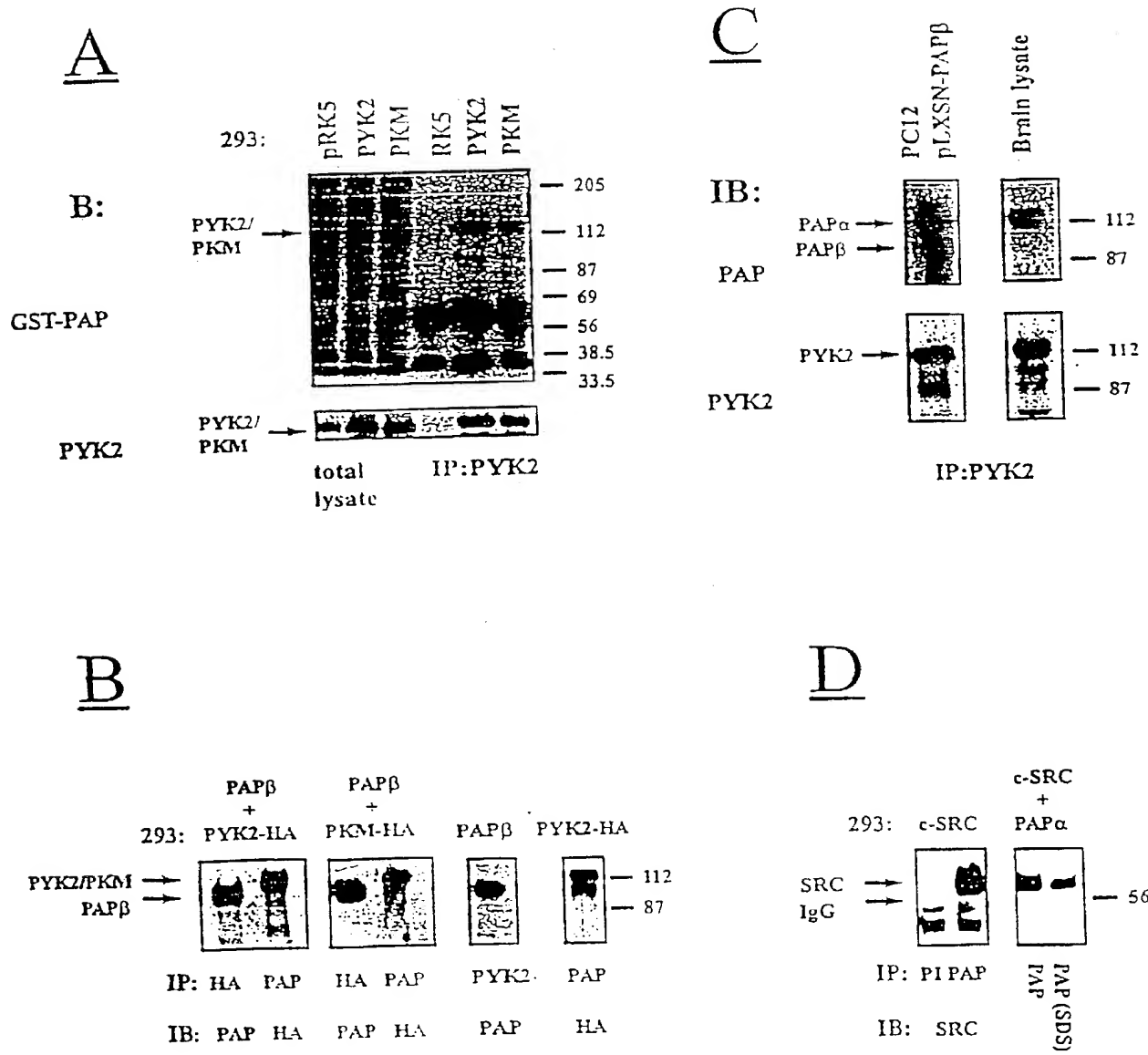
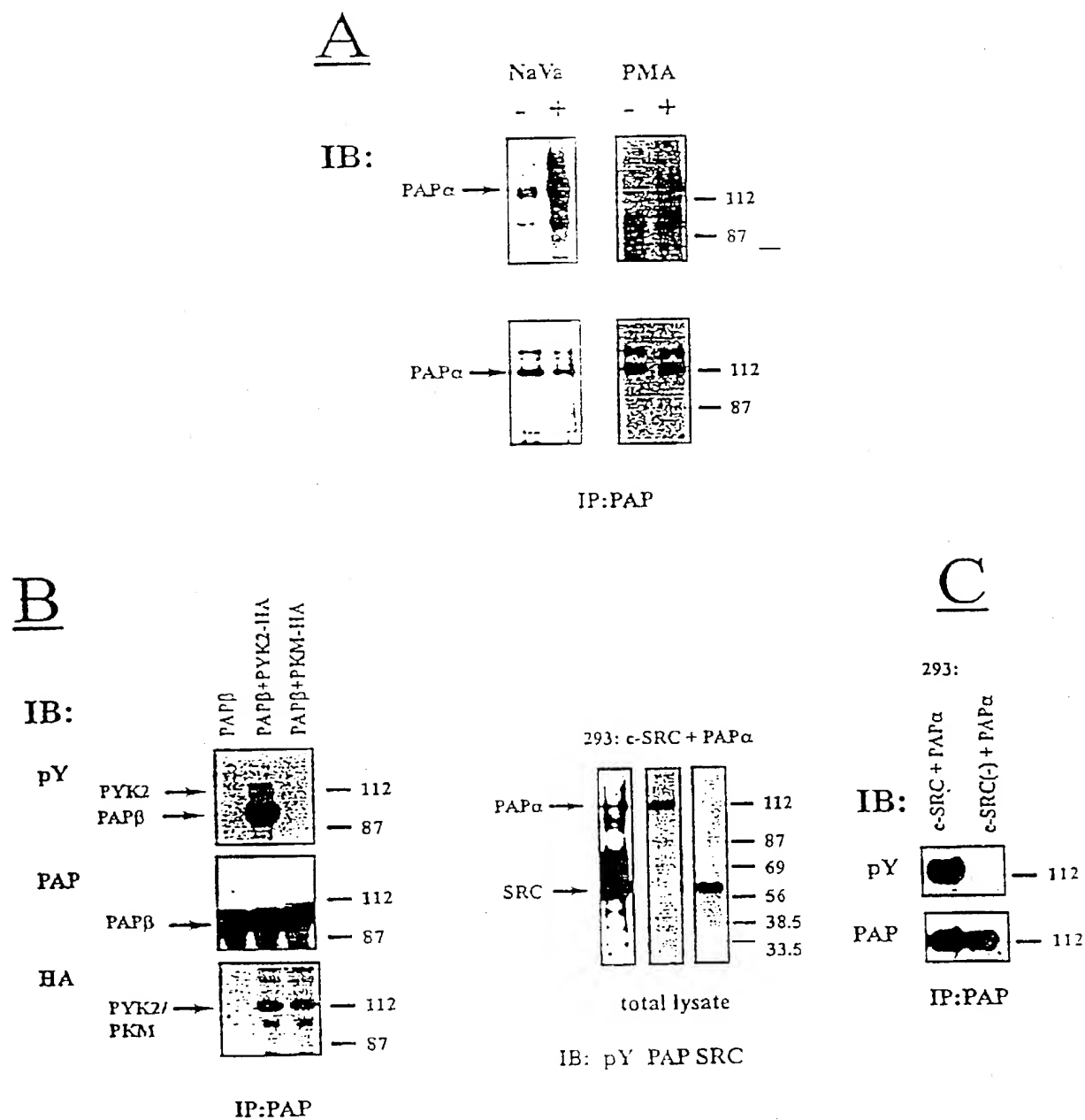


FIGURE 5



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FIGURE 6

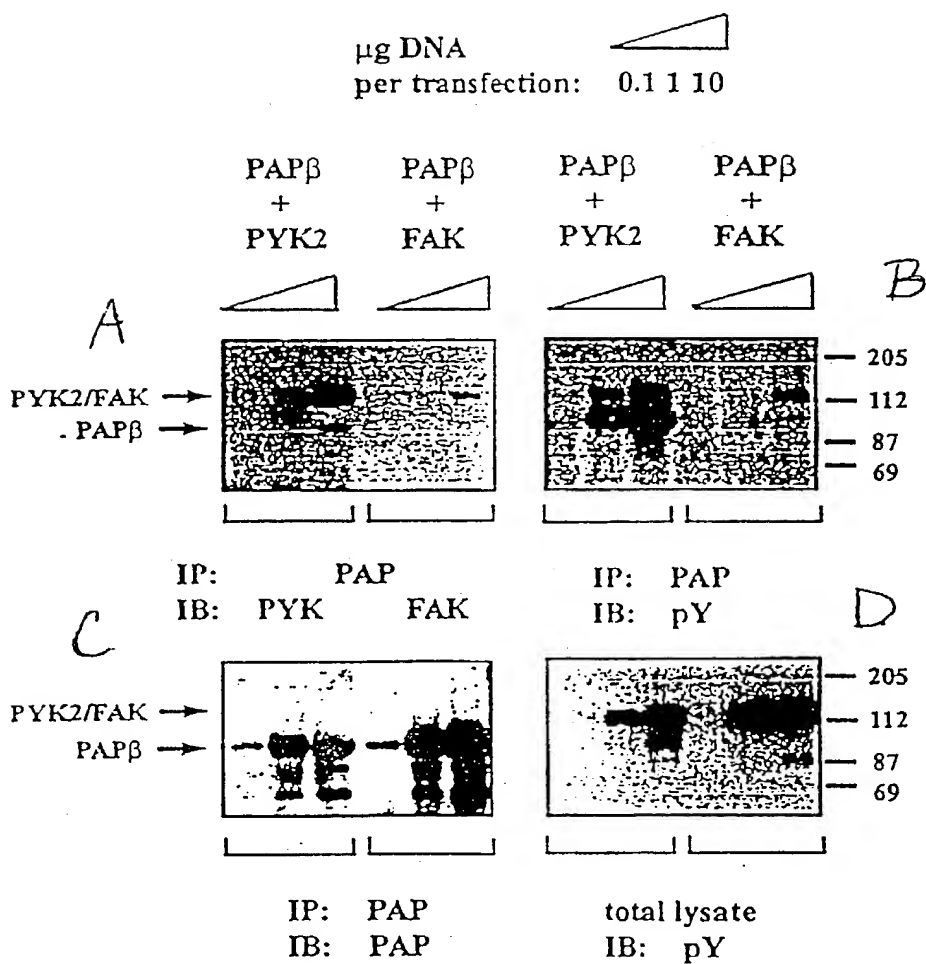


FIGURE 7

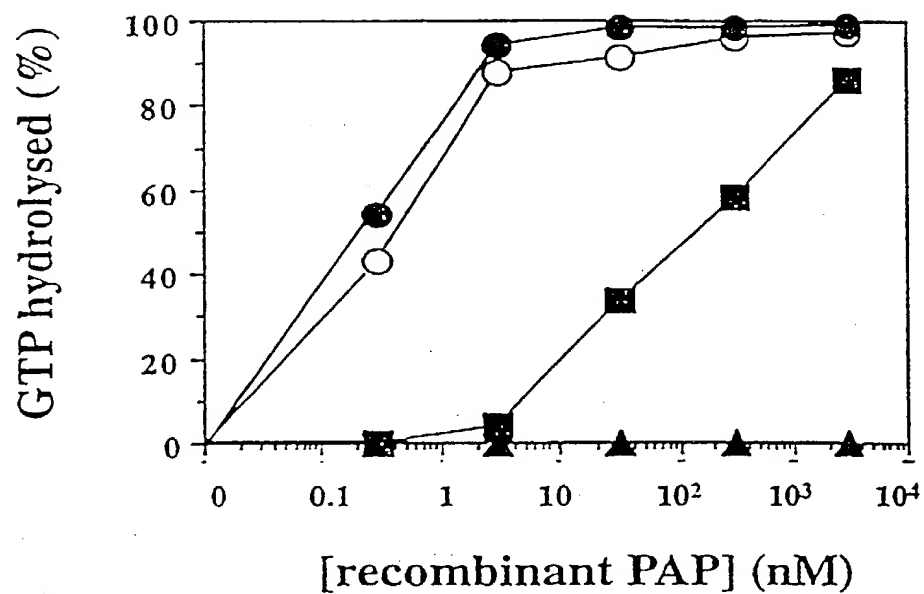
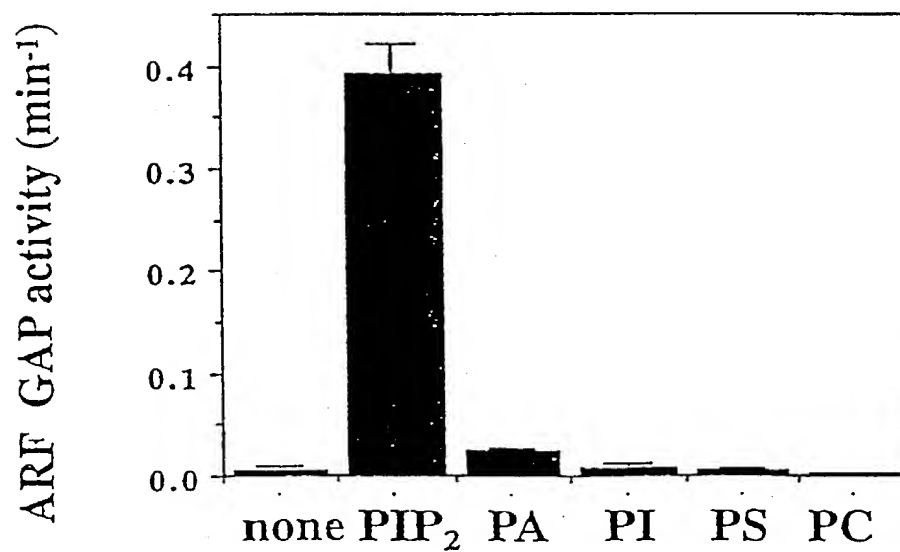
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FIGURE 8

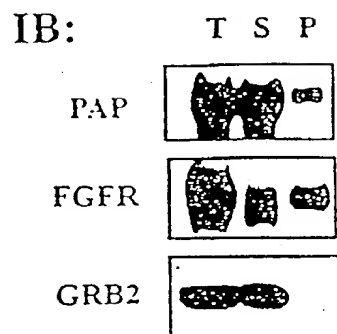
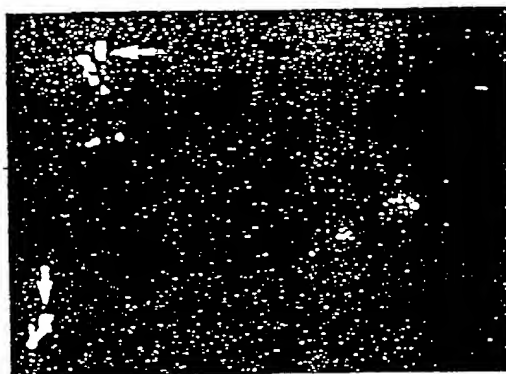


FIGURE 9

HeLa: PAP $\alpha$ -myc



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FIGURE 10

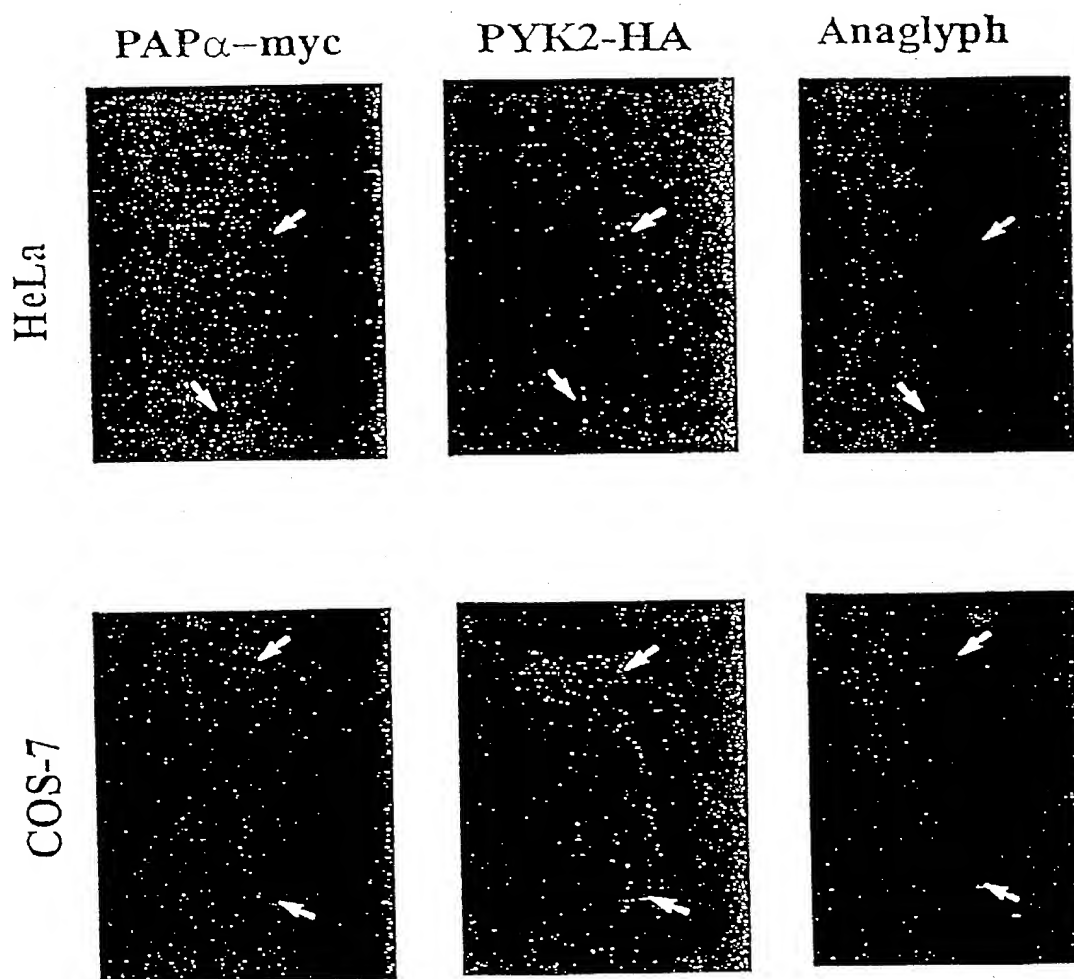
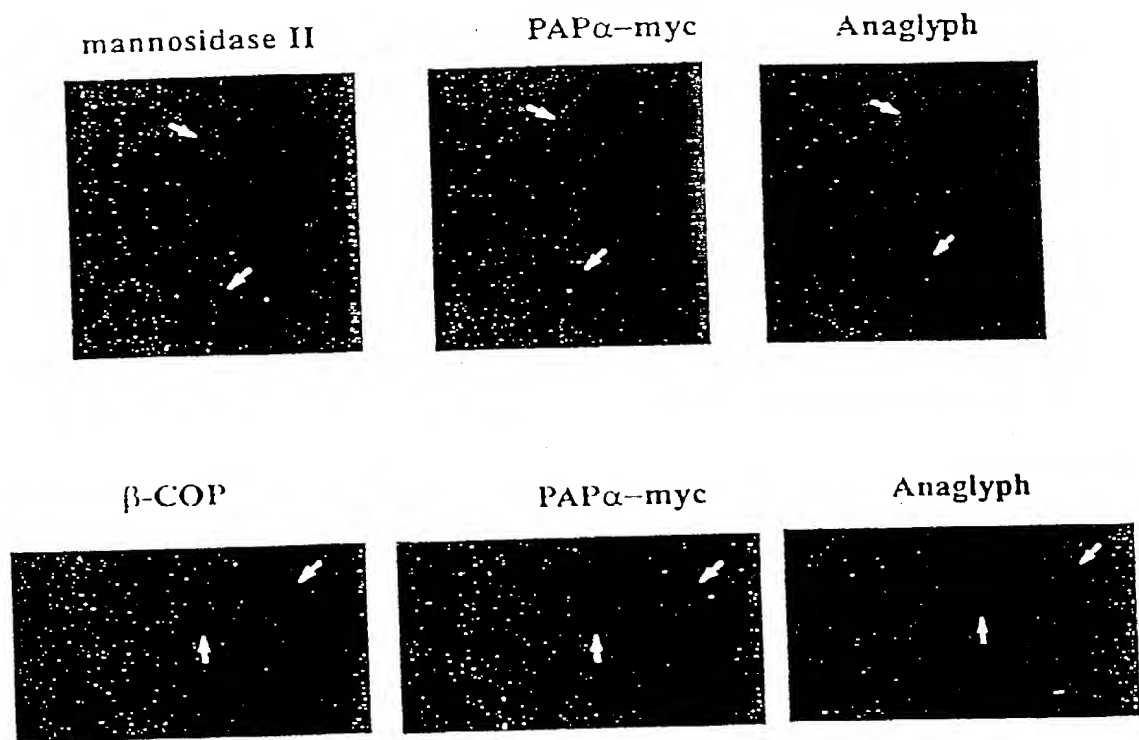


FIGURE 11



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FIGURE 12

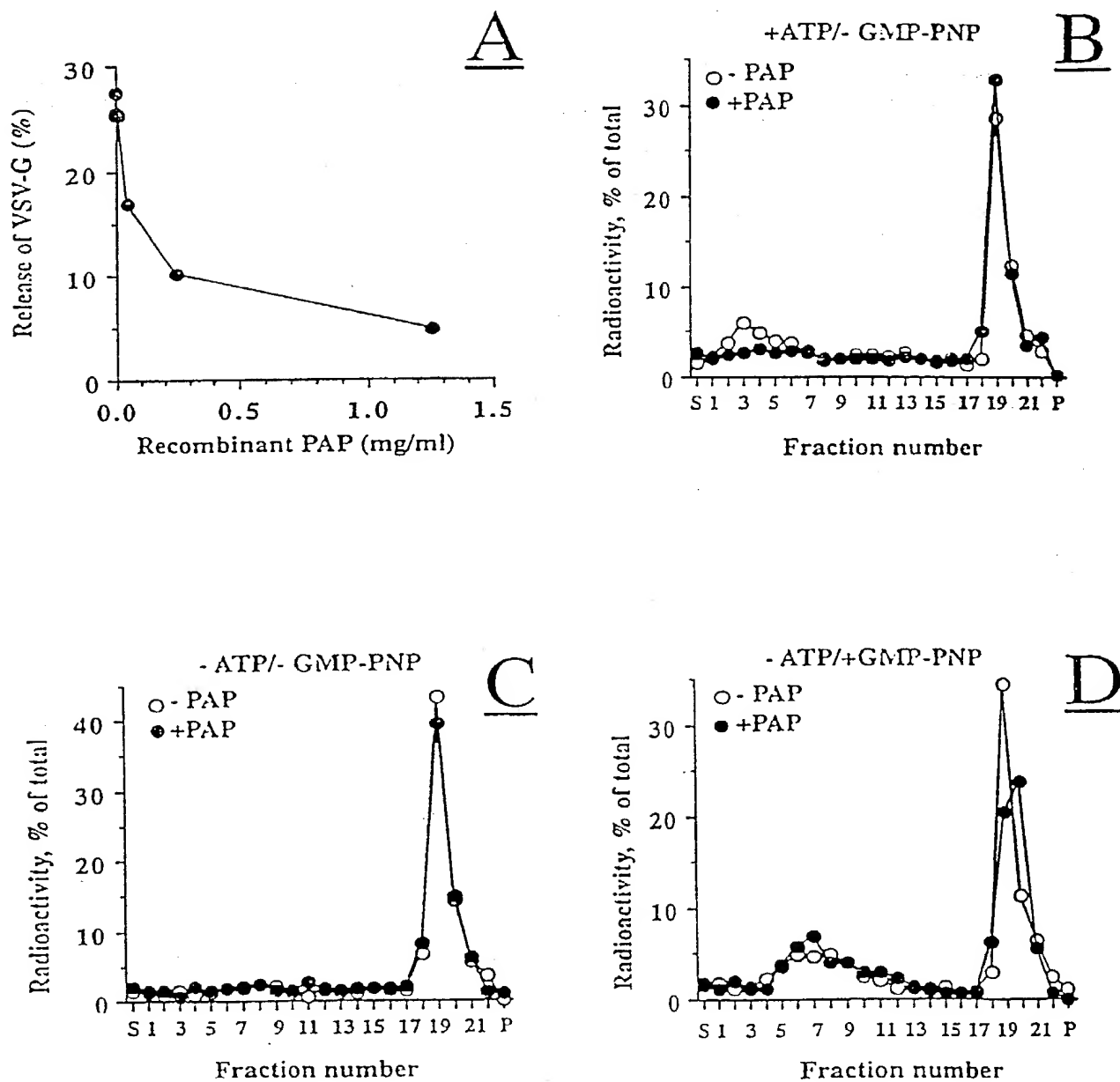
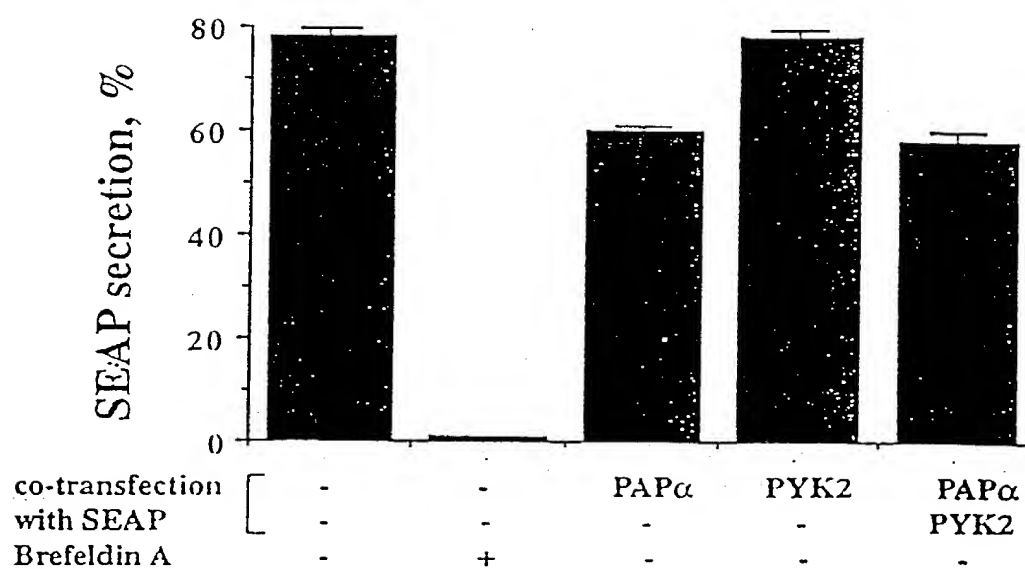


FIGURE 13



**DECLARATION AND POWER OF ATTORNEY**

As a below named inventor, I HEREBY DECLARE:

THAT my residence, post office address, and citizenship are as stated below next to my name;

THAT I believe I am the original, first, and sole inventor (if only one inventor is named below) or an original, first, and joint inventor (if plural inventors are named below or in an attached Declaration) of the subject matter which is claimed and for which a patent is sought on the invention entitled

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PYK2 BINDING PROTEINS

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(Attorney Docket No. 038602-1224)

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the specification of which (check one)

\_\_\_\_\_ is attached hereto.

  X   was filed on 08/22/2001 as United States Application Number 09/914,042 based on PCT International Application Number PCT/US00/04647 filed 2/20/2000 and was amended on \_\_\_\_\_ (if applicable).

THAT I do not know and do not believe that the same invention was ever known or used by others in the United States of America, or was patented or described in any printed publication in any country, before I (we) invented it;

THAT I do not know and do not believe that the same invention was patented or described in any printed publication in any country, or in public use or on sale in the United States of America, for more than one year prior to the filing date of this United States application;

THAT I do not know and do not believe that the same invention was first patented or made the subject of an inventor's certificate that issued in any country foreign to the United States of America before the filing date of this United States application if the foreign application was filed by me (us), or by my (our) legal representatives or assigns, more than twelve months (six months for design patents) prior to the filing date of this United States application;

THAT I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment specifically referred to above;

THAT I believe that the above-identified specification contains a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention, and sets forth the best mode contemplated by me of carrying out the invention; and

THAT I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I HEREBY CLAIM foreign priority benefits under Title 35, United States Code § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number	Country	Foreign Filing Date	Priority Claimed?	Certified Copy Attached?

I HEREBY CLAIM the benefit under Title 35, United States Code § 119(e) of any United States provisional application(s) listed below.

U.S. Provisional Application Number	Filing Date
60/121,125	02/22/1999

I HEREBY CLAIM the benefit under Title 35, United States Code, § 120 of any United States application(s), or § 365(c) of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application Number	PCT Parent Application Number	Parent Filing Date	Parent Patent Number

I HEREBY APPOINT the registered attorneys and agents at Customer Number 22428



22428

PATENT TRADEMARK OFFICE

to have full power to prosecute this application and any continuations, divisions, reissues, and reexaminations thereof, to receive the patent, and to transact all business in the United States Patent and Trademark Office connected therewith.



Atty. Dkt. No. 038602-1224

I request that all correspondence be directed to:

Beth A. Burrous  
 FOLEY & LARDNER  
 Customer Number: 22428



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I UNDERSTAND AND AGREE THAT the foregoing attorneys and agents appointed by me to prosecute this application do not personally represent me or my legal interests, but instead represent the interests of the legal owner(s) of the invention described in this application.

I FURTHER DECLARE THAT all statements made herein of my own knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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Inventor's signature	<u>J. Schlessinger</u>
Date	<u>3/2/02</u>
Name of second inventor	<u>Julian Andreev</u>
Residence	<u>Weymouth, Massachusetts</u>
Citizenship	<u>U.S.A.</u>
Post Office Address	<u>80 Old Stone Way, A305, Weymouth, Massachusetts 02189</u>
Inventor's signature	<u></u>
Date	<u></u>

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Citizenship	U.S.A.
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Inventor's signature	
Date	
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Residence	<u>Weymouth, Massachusetts</u> <i>MA</i>
Citizenship	U.S.A.
Post Office Address	80 Old Stone Way, A305, Weymouth, Massachusetts 02189
Inventor's signature	<i>J. Andreev</i>
Date	<i>02/28/02</i>

09/914042

1

SEQUENCE LISTING

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ANDREEV, JULIAN

<120> PYK2 BINDING PROTEINS

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<141> 2001-08-22

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 Asp Lys Ser Thr Pro Leu Thr Asn Lys Gly Gln Pro Arg Gly Pro Val  
 900 905 910  
 Asp Leu Ser Ala Thr Glu Ala Leu Gly Pro Leu Ser Asn Ala Met Val  
 915 920 925  
 Leu Gln Pro Pro Ala Pro Met Pro Arg Lys Ser Gln Ala Thr Lys Leu  
 930 935 940  
 Lys Pro Lys Arg Val Lys Ala Leu Tyr Asn Cys Val Ala Asp Asn Pro  
 945 950 955 960  
 Asp Glu Leu Thr Phe Ser Glu Gly Asp Val Ile Ile Val Asp Gly Glu  
 965 970 975  
 Glu Asp Gln Glu Trp Trp Ile Gly His Ile Asp Gly Asp Pro Gly Arg  
 980 985 990  
 Lys Gly Ala Phe Pro Val Ser Phe Val His Phe Ile Ala Asp  
 995 1000 1005  
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 Lys Glu Arg Arg Phe Phe Gln Leu Gln Met Cys Glu Tyr Leu Leu Lys  
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Val Asn Glu Ile Lys Val Lys Lys Gly Val Asp Leu Leu Gln Asn Leu  
 35 40 45  
 Ile Lys Tyr Phe His Ala Gln Cys Asn Phe Phe Gln Asp Gly Leu Lys  
 50 55 60  
 Ala Val Glu Ser Leu Lys Pro Ser Ile Glu Thr Leu Ser Thr Asp Leu  
 65 70 75 80  
 His Thr Ile Lys Gln Ala Gln Asp Glu Glu Arg Arg Gln Leu Ile Gln  
 85 90 95  
 Leu Arg Asp Ile Leu Lys Ser Ala Leu Gln Val Glu Gln Lys Glu Asp  
 100 105 110  
 Ser Gln Ile Arg Gln Ser Thr Ala Tyr Ser Leu His Gln Pro Gln Gly  
 115 120 125  
 Asn Lys Glu His Gly Thr Glu Arg Asn Gly Asn Leu Tyr Lys Lys Ser  
 130 135 140  
 Asp Gly Ile Arg Lys Val Trp Gln Lys Arg Lys Cys Ser Val Lys Asn  
 145 150 155 160  
 Gly Phe Leu Thr Ile Ser His Gly Thr Ala Asn Arg Pro Pro Ala Lys  
 165 170 175  
 Leu Asn Leu Leu Thr Cys Gln Val Lys Thr Asn Pro Glu Glu Lys Lys  
 180 185 190  
 Cys Phe Asp Leu Ile Ser His Asp Arg Thr Tyr His Phe Gln Ala Glu  
 195 200 205  
 Asp Glu Gln Glu Cys Gln Ile Trp Met Ser Val Leu Gln Asn Ser Lys  
 210 215 220  
 Glu Glu Ala Leu Asn Asn Ala Phe Lys Gly Asp Asp Asn Thr Gly Glu  
 225 230 235 240  
 Asn Asn Ile Val Gln Glu Leu Thr Lys Glu Ile Ile Ser Glu Val Gln  
 245 250 255  
 Arg Met Thr Gly Asn Asp Val Cys Cys Asp Cys Gly Ala Pro Asp Pro  
 260 265 270  
 Thr Trp Leu Ser Thr Asn Leu Gly Ile Leu Thr Cys Ile Glu Cys Ser  
 275 280 285  
 Gly Ile His Arg Glu Leu Gly Val His Tyr Ser Pro Met Gln Ser Leu  
 290 295 300  
 Thr Leu Asp Val Leu Gly Thr Ser Glu Leu Leu Leu Ala Lys Asn Ile  
 305 310 315 320  
 Gly Asn Ala Gly Phe Asn Glu Ile Met Glu Cys Cys Leu Pro Ser Glu  
 325 330 335

6

Asp	Pro	Val	Lys	Pro	Asn	Pro	Gly	Ser	Asp	Met	Ile	Ala	Arg	Lys	Asp
			340					345					350		
Tyr	Ile	Thr	Ala	Lys	Tyr	Met	Glu	Arg	Arg	Tyr	Ala	Arg	Lys	Lys	His
		355					360					365			
Ala	Asp	Thr	Ala	Ala	Lys	Leu	His	Ser	Leu	Cys	Glu	Ala	Val	Lys	Thr
		370				375					380				
Arg	Asp	Ile	Phe	Gly	Leu	Leu	Gln	Ala	Tyr	Ala	Asp	Gly	Val	Asp	Leu
385					390					395					400
Thr	Glu	Lys	Ile	Pro	Leu	Ala	Asn	Gly	His	Glu	Pro	Asp	Glu	Thr	Ala
				405					410					415	
Leu	His	Leu	Ala	Val	Arg	Ser	Val	Asp	Arg	Thr	Ser	Leu	His	Ile	Val
			420					425					430		
Asp	Phe	Leu	Val	Gln	Asn	Ser	Gly	Asn	Leu	Asp	Lys	Gln	Thr	Gly	Lys
		435					440					445			
Gly	Ser	Thr	Ala	Leu	His	Tyr	Cys	Cys	Leu	Thr	Asp	Asn	Ala	Glu	Cys
		450				455					460				
Leu	Lys	Leu	Leu	Leu	Arg	Gly	Lys	Ala	Ser	Ile	Glu	Ile	Ala	Asn	Glu
465					470					475					480
Ser	Gly	Glu	Thr	Pro	Leu	Asp	Ile	Ala	Lys	Arg	Leu	Lys	His	Glu	His
				485					490					495	
Cys	Glu	Glu	Leu	Leu	Thr	Gln	Ala	Leu	Ser	Gly	Arg	Phe	Asn	Ser	His
			500					505					510		
Val	His	Val	Glu	Tyr	Glu	Trp	Arg	Leu	Leu	His	Glu	Asp	Leu	Asp	Glu
			515				520					525			
Ser	Asp	Asp	Asp	Val	Asp	Glu	Lys	Leu	Gln	Pro	Ser	Glu	Asn	Arg	Arg
						535					540				
Glu	Asp	Arg	Pro	Val	Ser	Phe	Tyr	Gln	Leu	Gly	Ser	Ser	Gln	Phe	Gln
545					550					555					560
Ser	Asn	Ala	Val	Ser	Leu	Ala	Arg	Asp	Thr	Ala	Asn	Leu	Thr	Lys	Asp
				565					570					575	
Lys	Gln	Arg	Gly	Phe	Gly	Pro	Ser	Ile	Leu	Gln	Asn	Glu	Thr	Tyr	Gly
			580					585					590		
Ala	Ile	Leu	Ser	Gly	Ser	Pro	Pro	Ser	Ser	Gln	Ser	Ile	Pro	Pro	Ser
			595				600					605			
Thr	Thr	Ser	Ala	Pro	Pro	Leu	Pro	Pro	Arg	Asn	Val	Gly	Lys	Asp	Pro
						615					620				
Leu	Thr	Thr	Thr	Pro	Pro	Pro	Pro	Val	Ala	Lys	Thr	Ser	Gly	Thr	Leu
625					630					635					640



7

Glu Ala Met Asn Gln Pro Ser Lys Ser Ser Gln Pro Gly Thr Ser Gln  
                     645                    650                    655  
 Ser Lys Pro Pro Pro Leu Pro Pro Gln Pro Pro Ser Arg Leu Pro Gln  
                     660                    665                    670  
 Lys Lys Pro Ala Ser Gly Ala Asp Lys Pro Thr Pro Leu Thr Asn Lys  
                     675                    680                    685  
 Gly Gln Pro Arg Gly Pro Glu Ala Ser Gly Pro Leu Ser Asn Ala Met  
                     690                    695                    700  
 Ala Leu Gln Pro Pro Ala Pro Met Pro Arg Lys Ser Gln Ala Thr Lys  
                     705                    710                    715                    720  
 Ser Lys Pro Lys Arg Val Lys Ala Leu Tyr Asn Cys Val Ala Asp Asn  
                     725                    730                    735  
 Pro Glu Ala Ser Gly Pro Leu Ser Asn Ala Met Ala Leu Gln Pro Pro  
                     740                    745                    750  
 Ala Pro Met Pro Arg Lys Ser Gln Ala Thr Lys Ser Lys Pro Lys Arg  
                     755                    760                    765  
 Val Lys Ala Leu Tyr Asn Cys Val Ala Asp Asn Pro Asp Glu Leu Thr  
                     770                    775                    780  
 Phe Ser Glu Gly Asp Val Ile Ile Val Asp Gly Glu Glu Asp Gln Glu  
                     785                    790                    795                    800  
 Trp Trp Ile Gly His Ile Asp Gly Glu Pro Ser Arg Lys Gly Ala Phe  
                     805                    810                    815  
 Pro Val Ser Phe Val His Phe Ile Ala Asp  
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&lt;210&gt; 5

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&lt;212&gt; PRT

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&lt;400&gt; 5

 Met Ala Ser Pro Arg Thr Arg Lys Val Leu Lys Glu Val Arg Ala Gln  
                     1                    5                    10                    15

 Asp Glu Asn Asn Val Cys Phe Glu Cys Gly Ala Phe Asn Pro Trp Val  
                     20                    25                    30

8

Ser Val Thr Tyr Gly Ile Trp Ile Cys Leu Glu Cys Ser Gly Arg His  
35 40 45

Arg Gly Leu Gly Val His Leu Ser Phe Val Arg Ser Val Thr Met Asp  
50 55 60

Lys Trp Lys Asp Ile Glu Leu Glu Lys Met Lys Ala Gly Gly Asn Ala  
65 70 75 80

Lys Phe Arg Glu Phe Leu Glu Ala Gln Asp Asp Tyr Glu Pro Ser Trp  
85 90 95

Ser Leu Asp Lys Tyr Ser Ser Arg Ala Ala Ala Leu Phe Arg Asp Lys  
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Val Ala Thr Leu Ala Glu Gly  
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<211> 117

<212> PRT

<213> Homo sapiens

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Lys Val Asp Pro Asp Thr Arg Arg Arg Leu Leu Gln Leu Gln Lys Ile  
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Gly Ala Asn Lys Lys Cys Met Asp Cys Gly Ala Pro Asn Pro Trp Ala  
20 25 30

Thr Pro Lys Phe Gly Ala Phe Ile Cys Leu Glu Cys Ala Gly Ile His  
35 40 45

Arg Gly Leu Gly Val His Phe Val Arg Ser Ile Thr Met Asp Gln Phe  
50 55 60

Lys Pro Glu Glu Leu Leu Arg Met Glu Lys Gly Gly Asn Glu Pro Leu  
65 70 75 80

Thr Glu Trp Phe Lys Ser His Asn Ile Asp Leu Ser Leu Pro Gln Lys  
85 90 95

Val Lys Tyr Asp Asn Pro Val Ala Glu Asp Tyr Lys Glu Lys Leu Thr  
100 105 110

Cys Leu Cys Glu Asp  
115

<210> 7

<211> 138

<212> PRT

<213> Mus musculus

<400> 7

Ile Val Gln Glu Leu Thr Lys Glu Ile Ile Ser Glu Val Arg Met Thr  
1 5 10 15

9

Gly Asn Asp Val Cys Cys Asp Cys Gly Ala Pro Asp Pro Thr Trp Leu  
 20 25 30  
 Ser Thr Asn Leu Gly Ile Leu Thr Cys Ile Glu Cys Ser Gly Ile His  
 35 40 45  
 Arg Glu Leu Gly Val His Tyr Ser Arg Met Gln Ser Leu Thr Leu Asp  
 50 55 60  
 Val Leu Gly Thr Ser Glu Leu Leu Leu Ala Lys Asn Ile Gly Asn Ala  
 65 70 75 80  
 Gly Phe Asn Glu Ile Met Glu Cys Cys Leu Pro Ser Glu Asp Pro Val  
 85 90 95  
 Lys Pro Asn Pro Gly Ser Asp Met Ile Ala Arg Lys Asp Tyr Ile Thr  
 100 105 110  
 Ala Lys Tyr Met Glu Arg Arg Tyr Ala Arg Lys Lys His Ala Asp Thr  
 115 120 125  
 Ala Ala Lys Leu His Ser Leu Cys Glu Ala  
 130 135

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<223> Description of Artificial Sequence: Zinc finger motif

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<222> (2)..(3)

<223> Variable amino acid

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<222> (5)..(20)

<223> Variable amino acid

<220>

<221> MOD\_RES

<222> (22)..(23)

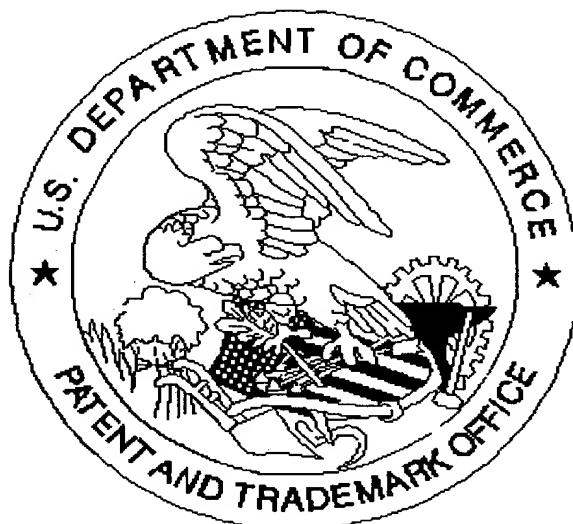
<223> Variable amino acid

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Cys Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa  
 1 5 10 15

Xaa Xaa Xaa Xaa Cys Xaa Xaa Cys  
 20

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